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(71) Applicant *(for all designated States except US)*: **FIBROGEN, INC.** [US/US]; 225 Gateway Boulevard, South San Francisco, CA 94080 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **GUENZLER-PUKALL, Volkmar** [DE/US]; 1408 Regent Drive, San Leandro, CA 94577 (US). **NEFF, Thomas, B.** [US/US]; 190 Glenwood Avenue, Atherton, CA 94027 (US). **WANG, Qingjian** [US/US]; 2115 Everglades Place, Davis, CA 95616 (US). **AREND, Michael, P.** [DE/US]; 36 East 40th Avenue, Apartment #E, San Mateo, CA 94403 (US). **FLIPPIN, Lee, A.** [US/US]; 17659 Skyline Boulevard, Woodside, CA 94062 (US). **MELEKHOV, Alex** [RU/US]; 2153 Vista Del Mar, San Mateo, CA 94404 (US).

(74) Agents: **PRICE, Leanne, C.** et al.; Intellectual Property Department, Fibrogen, Inc., 225 Gateway Boulevard, South San Francisco, CA 94080 (US).

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(54) Title: STABILIZATION OF HYPOXIA INDUCIBLE FACTOR (HIF) ALPHA

(57) Abstract: The present invention relates to methods of stabilizing the alpha subunit of hypoxia inducible factor (HIF). The invention further relates to methods of preventing, pretreating, or treating conditions associated with HIF, including ischemic and hypoxic conditions. Compounds for use in these methods are also provided.

# STABILIZATION OF HYPOXIA INDUCIBLE FACTOR (HIF) ALPHA

[0001] This application claims the benefit of U.S. Provisional Application Serial No. 60/337,082, filed on 6 December 2001; U.S. Provisional Application Serial No. 60/359,683, filed on 25 February 2002; U.S. Provisional Application Serial No. 60/349,659, filed on 16 January 2002; and U.S. Provisional Application Serial No. 60/386,488, filed on 5 June 2002, each of which is incorporated by reference herein in its entirety.

## FIELD OF THE INVENTION

[0002] The present invention relates to methods of stabilizing the alpha subunit of hypoxia inducible factor (HIF) and to compounds that can be used in these methods.

## BACKGROUND OF THE INVENTION

[0003] An early response to tissue hypoxia is induction of hypoxia inducible factor (HIF), a basic helix-loop-helix (bHLH) PAS (Per/Arnt/Sim) transcriptional activator that mediates changes in gene expression in response to changes in cellular oxygen concentration. HIF is a heterodimer containing an oxygen-regulated alpha subunit (HIF$\alpha$) and a constitutively expressed beta subunit (HIF$\beta$), also known as aryl hydrocarbon receptor nuclear transporter (ARNT). In oxygenated (normoxic) cells, HIF$\alpha$ subunits are rapidly degraded by a mechanism that involves ubiquitination by the von Hippel-Lindau tumor suppressor (pVHL) E3 ligase complex. Under hypoxic conditions, HIF$\alpha$ is not degraded, and an active HIF$\alpha/\beta$ complex accumulates in the nucleus and activates the expression of several genes including glycolytic enzymes, glucose transporter (GLUT)-1, erythropoietin (EPO), and vascular endothelial growth factor (VEGF). (Jiang et al. (1996) J Biol Chem 271:17771-17778; Iliopoulus et al. (1996) Proc Natl Acad Sci USA 93:10595-10599; Maxwell et al. (1999) Nature 399:271-275; Sutter et al. (2000) Proc Natl Acad Sci USA 97:4748-4753; Cockman et al. (2000) J Biol Chem 275:25733-25741; and Tanimoto et al. (2000) EMBO J 19:4298-4309.)

[0004] Levels of HIF$\alpha$ protein are elevated in most cells in response to hypoxia and HIF$\alpha$ is induced *in vivo* when animals are subjected to anemia or hypoxia. HIF$\alpha$ levels rise within a few hours after the onset of hypoxia and return to baseline under continued hypoxic conditions. HIF has been implicated in numerous cellular and developmental processes including cell proliferation, angiogenesis, and cell cycle arrest. HIF$\alpha$ has also been associated with myocardial acute ischemia and early infarction, pulmonary hypertension, and inflammation. Although HIF$\alpha$ has been associated with tumor growth and metastasis, there is little indication that HIF is directly involved in tumorigenesis. Hypoxic preconditioning, in

which a target organ is subjected to brief periods of hypoxia, has been shown to protect both myocardium and brain against hypoxic-ischemic injury. HIFα stabilization is closely associated with ischemia and is induced by preconditioning. (Wang and Semenza (1993) Proc Natl Acad Sci USA 90:4304-4308; Stroka et al. (2001) FASEB J 15:2445-2453; Semenza et al. (1997) Kidney Int 51:553-555; Carmeliet et al. (1998) Nature 394:485-490; Zhong et al. (1999) Cancer Res 59:5830-5835; Lee et al. (2000) N Engl J Med 343:148-149; Sharp et al. (2000) J Cereb Blood Flow Metab 20:1011-1032; Semenza et al. (2000) Adv Exp Med Biol 475:123-130; Thornton et al. (2000) Biochem J 350:307-312; Deindl and Schaper (1998) Mol Cell Biochem 186:43-51; Bergeron et al. (2000) Ann Neurol 48:285-296.)

[0005] Several investigators have studied the mechanism of interaction between HIFα and pVHL. An oxygen-dependent degradation domain (ODD) within HIF-1α from residue 401 to 603 was originally identified as sufficient to confer oxygen-dependent instability to chimeric protein constructs. A domain containing a portion of the ODD, from residue 526 to 652, was found to be required for pVHL-dependent degradation. Further, mutation of $P_{564}YI$ to aspartic acids or mutation of $K_{532}$ to arginine within a region conserved among HIFα homologs (residue 556 to 574 in HIF-1α) rendered the full-length HIFα protein stable under normoxic conditions and resistant to pVHL-mediated degradation. (Huang et al. (1998) Proc Natl Acad Sci USA 95:7987-7992; and Tanimoto et al. (2000) EMBO J 19:4298-4309.)

[0006] HIFα levels are increased by a number of factors that mimic hypoxia, including iron chelators such as desferrioxamine (DFO) and divalent metal salts such as $CoCl_2$. HIFα levels are increased by angiotensin II, thrombin, and platelet-derived growth factor under normoxic conditions using a mechanism involving reactive oxygen species. Reports have also suggested HIFα is regulated by phosphorylation through pathways involving nitric oxide-activated phosphotidylinositol 3′-kinase (PI3K), hepatocyte growth factor, or mitogen-activated protein kinase. Glycogen-synthase kinase, which is a downstream target of PI3K, directly phosphorylates the HIFα ODD domain. (Richard et al. (2000) J Biol Chem 275:26765-26771; Sandau et al. (2000) Biochem Biophys Res Commun 278:263-267; Tacchini et al. (2001) Carcinogenesis 22:1363-1371; and Sodhi et al. (2001) Biochem Biophys Res Commun 287:292-300.)

[0007] Hypoxia, a state of reduced oxygen, can occur when the lungs are compromised or blood flow is reduced. Ischemia, reduction in blood flow, can be caused by the obstruction of an artery or vein by a blood clot (thrombus) or by any foreign circulating matter (embolus), or by a vascular disorder such as atherosclerosis. Reduction in blood flow

can have a sudden onset and short duration (acute ischemia), or can have a slow onset with long duration or frequent recurrence (chronic ischemia). Acute ischemia is often associated with regional, irreversible tissue necrosis (an infarct), whereas chronic ischemia is usually associated with transient hypoxic tissue injury. If the decrease in perfusion is prolonged or severe, however, chronic ischemia can also be associated with an infarct. Infarctions commonly occur in the spleen, kidney, lungs, brain, and heart, producing disorders such as intestinal infarction, pulmonary infarction, ischemic stroke, and myocardial infarction.

[0008] Pathologic changes in ischemic disorders depend on the duration and severity of ischemia, and on the length of patient survival. Necrosis can be seen within the infarct in the first 24 hours, and an acute inflammatory response develops in the viable tissue adjacent to the infarct with leukocytes migrating into the area of dead tissue. Over succeeding days, there is a gradual breakdown and removal of cells within the infarct by phagocytosis, and replacement with a collagenous or glial scar.

[0009] Hypoperfusion or infarction in one organ often affects other organs. For example, ischemia of the lung, caused by, for example, a pulmonary embolism, not only affects the lung, but also puts the heart and other organs, such as the brain, under hypoxic stress. Myocardial infarction, which often involves coronary artery blockage due to thrombosis, arterial wall vasospasms, or viral infection of the heart, can lead to congestive heart failure and systemic hypotension. Secondary complications such as global ischemic encephalopathy can develop if the cardiac arrest is prolonged with continued hypoperfusion. Cerebral ischemia, most commonly caused by vascular occlusion due to atherosclerosis, can range in severity from transient ischemic attacks (TIAs) to cerebral infarction or stroke. While the symptoms of TIAs are temporary and reversible, TIAs tend to recur and are often followed by a stroke.

[0010] Occlusive arterial disease includes coronary artery disease, which can lead to myocardial infarction, and peripheral arterial disease, which can affect the abdominal aorta, its major branches, and arteries of the legs. Peripheral arterial disease includes Buerger's disease, Raynaud's disease, and acrocyanosis. Although peripheral arterial disease is commonly caused by atherosclerosis, other major causes include, e.g., diabetes, etc. Complications associated with peripheral arterial disease include severe leg cramps, angina, abnormal heart rhythms, heart failure, heart attack, stroke, and kidney failure.

[0011] Ischemic and hypoxic disorders are a major cause of morbidity and mortality. Cardiovascular diseases cause at least 15 million deaths every year and are responsible for

30% of deaths worldwide. Among the various cardiovascular diseases, ischemic heart disease and cerebrovascular diseases cause approximately 17% of deaths. Annually, 1.3 million cases of nonfatal acute myocardial infarction are reported, making the prevalence approximately 600 per 100,000 people. Further, an estimated five million Americans suffer from venous thrombosis every year, and approximately 600,000 of these cases result in pulmonary embolism. About one-third of the pulmonary embolisms end in death, making pulmonary embolism the third most common cause of death in the United States.

[0012] Currently, treatment of ischemic and hypoxic disorders is focused on relief of symptoms and treatment of causative disorders. For example, treatments for myocardial infarction include nitroglycerin and analgesics to control pain and relieve the workload of the heart. Other medications, including digoxin, diuretics, amrinone, ß-blockers, lipid-lowering agents and angiotensin-converting enzyme inhibitors, are used to stabilize the condition, but none of these therapies directly address the tissue damage produced by the ischemia and hypoxia.

[0013] Due to deficiencies in current treatments, there remains a need for methods that are effective in treating conditions involving ischemia and hypoxia such as occlusive arterial disease, angina pectoris, intestinal infarctions, pulmonary infarctions, cerebral ischemia, and myocardial infarction. There is also a need for methods that are effective in the prevention of tissue damage caused by ischemia that occurs due to, e.g., atherosclerosis, diabetes, and pulmonary disorders such as pulmonary embolism and the like. In summary, there is a need in the art for methods and compounds that can be used to stabilize HIF, and to treat and prevent HIF-associated disorders including conditions involving ischemia and hypoxia.

## SUMMARY OF THE INVENTION

[0014] Described herein are methods of stabilizing the alpha subunit of hypoxia inducible factor (HIFα). These methods can be applied *in vivo* or *in vitro*.

[0015] The present invention relates generally to methods of stabilizing the alpha subunit of hypoxia inducible factor (HIF). In one embodiment, the method of stabilizing the alpha subunit of HIF (HIFα) comprises administering to a subject a compound that inhibits hydroxylation of HIFα. In certain of the embodiments of the present invention, the HIFα is selected from the group consisting of HIF-1α, HIF-2α, HIF-3α, and any fragment thereof. In a further embodiment, the method comprises administering to a subject a compound that inhibits 2-oxoglutarate dioxygenase enzyme activity. In various embodiments, the

2-oxoglutarate dioxygenase enzyme is selected from the group consisting of EGLN1, EGLN2, EGLN3, procollagen prolyl 4-hydroxylase, procollagen prolyl 3-hydroxylase, procollagen lysyl hydroxylase, PHD4, FIH-1, and any subunit or fragment thereof, respectively.

[0016] In particular methods for stabilizing HIFα according to the present invention, the methods comprise inhibiting HIF prolyl hydroxylase enzyme activity. In further embodiments, the HIF prolyl hydroxylase enzyme is selected from the group consisting of EGLN1, EGLN2, EGLN3, and any subunit or fragment thereof, respectively.

[0017] The present invention provides, in one aspect, methods for stabilizing endogenous HIFα. Thus, in a particular embodiment, the HIFα is endogenous to the subject. Embodiments of the present invention include methods for stabilizing HIFα in which a compound that stabilizes HIFα is administered to a subject *in vivo*. The subject can be, for example, an animal, preferably, a mammal, and, more preferably, a human. Methods of *ex vivo* administration are also contemplated. In such methods, the subject can be, e.g., a cell, tissue, or organ, etc. In certain embodiments, the subject is a cell, tissue, or organ derived from a system such as the renal, cardiac, hepatic, pulmonary, hematopoietic, gastrointestinal, neuronal, or musculoskeletal system, etc.

[0018] Methods for treating, preventing, or pretreating a HIF-associated condition are also provided. In particular, the present invention provides a method for treating, preventing, or pretreating a HIF-associated condition, the method comprising stabilizing HIFα. In specific aspects, the invention provide a method for treatment, prevention, or pretreatment/ preconditioning of a HIF-associated condition in a subject, the method comprising stabilization of HIFα. In various aspects, the HIF-associated condition is associated with ischemia or hypoxia. In a preferred aspect, the method comprises administering to the subject a compound that stabilizes HIFα.

[0019] In various embodiments, the compound is selected from the group consisting of heterocyclic carboxamides, phenanthrolines, hydroxamates, and physiologically active salts and prodrugs derived therefrom. In particular embodiments, the compound is a heterocyclic carboxamide selected from the group consisting of pyridine carboxamides, quinoline carboxamides, isoquinoline carboxamides, cinnoline carboxamides, and beta-carboline carboxamides. In a preferred embodiment of the present invention, the compound is delivered in an oral formulation. In another preferred embodiment, the compound is delivered in a transdermal formulation.

[0020] In one method of stabilizing HIFα according to the present invention, the compound stabilizes HIFα by specifically inhibiting hydroxylation of at least one amino acid residue in HIFα. In a further aspect, the amino acid residue is selected from the group consisting of proline and asparagine.

[0021] Methods for treating, preventing, or pretreating a HIF-associated condition in a subject, the methods comprising inhibiting 2-oxoglutarate dioxygenase enzyme activity, are also provided, and include methods in which the HIF-associated condition is one associated with ischemia or hypoxia. In one aspect, the present invention provides a method for treating, preventing, or pretreating a HIF-associated condition, the method comprising administering to the subject a compound that inhibits 2-oxoglutarate dioxygenase enzyme activity.

[0022] In a preferred embodiment, the present invention provides a method of treating, preventing, or pretreating a HIF-associated condition in a subject, the method comprising inhibiting HIF prolyl hydroxylase enzyme activity. Again, HIF-associated conditions include those associated with hypoxia, or with ischemia, etc. In a particular embodiment, the method comprises administering to the subject a compound that inhibits HIF prolyl hydroxylase activity.

[0023] In a further embodiment, the method further comprises administering a second compound. In particular embodiments, the second compound inhibits 2-oxoglutarate dioxygenase enzyme activity, or the compound and the second compound inhibit the activities of different 2-oxoglutarate dioxygenase enzymes, or the second compound is selected from the group consisting of an ACE inhibitor (ACEI), angiotensin-II receptor blocker (ARB), diuretic, digoxin, statin, or carnitine, etc.

[0024] In specific embodiments, HIF-associated conditions include disorders such as pulmonary disorders, e.g., pulmonary embolism, etc., cardiac disorders, e.g., myocardial infarction, congestive heart failure, etc., neurological disorders, and the like. The present invention thus clearly contemplates methods that can be applied to the treatment, prevention, or pretreatment/preconditioning of a HIF-associated condition associated with any ischemic event, whether acute or transient, or chronic. Acute ischemic events can include those associated with surgery, organ transplantation, infarction (e.g., cerebral, intestinal, myocardial, pulmonary, etc.), trauma, insult, or injury, etc. Chronic events associated with ischemia can include hypertension, diabetes, occlusive arterial disease, chronic venous insufficiency, Raynaud's disease, cirrhosis, congestive heart failure, systemic sclerosis, etc.

[0025] Methods of preconditioning or pretreating are specifically contemplated. In one embodiment, the invention provides methods of pretreating or preconditioning wherein HIFα is stabilized prior to the occurrence of an event associated with a HIF-associated condition, e.g., ischemia, etc., or the development of a HIF-associated condition. Ischemias can be induced by acute events. Such events can include, for example, surgery, e.g., angioplasty, organ transplantation, etc., and related procedures such as administration of anesthesia, etc. Furthermore, chronic events specific embodiments, the methods of pretreating or preconditioning are applied in situations where a subject has a disorder predictive of the development of a HIF-associated condition, e.g., transient ischemic attack or angina pectoris, indicative of stroke and myocardial infarction, respectively, in order to prevent the development of or reduce the degree of development of the HIF-associated condition. In a particular embodiment, a compound that stabilizes HIFα is administered to a subject in order to increase preconditioning factors for ischemia, for example, EPO, etc.

[0026] Methods for increasing expression of various HIF-related factors are specifically contemplated herein. In one aspect, the present invention provides a method for increasing expression of angiogenic factors in a subject, the method comprising stabilizing HIFα. In another aspect, the present invention provides a method of increasing expression of glycolytic factors in a subject, the method comprising stabilizing HIFα. In a further aspect, the invention provides a method of increasing expression of factors associated with oxidative stress in a subject, the method comprising stabilizing HIFα. A method of treating a subject having a disorder associated with ischemic reperfusion injury, the method comprising stabilizing HIFα, is also contemplated.

[0027] Methods for identifying compounds that stabilize HIFα are also provided herein. For example, the present invention provides a method of identifying a compound that stabilizes HIFα, the method comprising: (a) administering a compound of interest to a subject or to a sample from a subject; (b) measuring the HIFα level in the subject or in the sample; and (c) comparing the HIFα level in the subject or in the sample to a standard level, wherein an increase in the HIFα level in the subject or the sample is indicative of a compound that stabilizes HIFα.

[0028] In another aspect, the methods of the invention are used to prevent the tissue damage caused by HIF-associated disorders including, but not limited to, ischemic and hypoxic disorders. In one embodiment, treatment is predicated on predisposing conditions,

e.g.,hypertension, diabetes, occlusive arterial disease, chronic venous insufficiency, Raynaud's disease, cirrhosis, congestive heart failure, and systemic sclerosis.

[0029] In yet another aspect, the methods of the invention can be used as a pretreatment to decrease or prevent the tissue damage caused by HIF-associated disorders including, but not limited to, ischemic and hypoxic disorders. In one embodiment, the need for pretreatment is based on a patient's history of recurring episodes of an ischemic condition, e.g., myocardial infarction or transient ischemic attacks, or has symptoms of impending ischemia, e.g., angina pectoris, etc. In another embodiment, the need for pretreatment is based on physical parameters implicating possible or likely ischemia or hypoxia, such as is the case with, e.g., individuals placed under general anesthesia or temporarily working at high altitudes. In yet another embodiment, the methods may be used in the context of organ transplants to pretreat organ donors and to maintain organs removed from the body prior to implantation in a recipient.

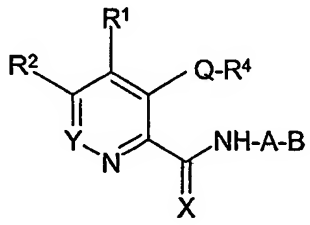
[0030] In another aspect, the invention provides compounds that stabilize HIF$\alpha$ and methods of using the compounds to prevent, pretreat, or treat HIF-associated conditions such as those described above. In one embodiment, a therapeutically effective amount of the compound or a pharmaceutically acceptable salt thereof, alone or in combination with a pharmaceutically acceptable excipient, is administered to a subject having a HIF-associated condition. In one specific embodiment, the compound is administered immediately following the diagnosis of an acute ischemic disorder. In another specific embodiment, the compound is administered to a subject during the course of a chronic ischemic condition. In yet another specific embodiment, the ischemia is due to a transient or acute trauma, insult, or injury such as, e.g., a spinal cord injury. In a specific embodiment, the compound is administered to a patient in need following diagnosis of a pulmonary disorder such as COPD and the like.

[0031] In one aspect, the compound can be administered based on predisposing conditions, e.g., chronic conditions, or as a pretreatment to decrease or prevent tissue damage caused by HIF-associated disorders. In a specific aspect, the compound is administered to a subject who has a history of recurring episodes of an ischemic condition, e.g., myocardial infarction or transient ischemic attacks, or has symptoms of impending ischemia, e.g.,angina pectoris. In another specific embodiment, the compound is administered based on physical parameters implicating possible ischemia or hypoxia, such as is the case with, e.g., individuals placed under general anesthesia or temporarily working at high altitudes. In yet another embodiment, the compounds may be used in the context of organ transplants to

pretreat organ donors and to maintain organs removed from the body prior to implantation in a recipient.

[0032] In one aspect, a compound of the present invention stabilizes HIFα by specifically inhibiting hydroxylation of amino acid residues in the HIFα protein. In one embodiment, the agent inhibits hydroxylation of HIFα proline residues. In one specific embodiment, the agent inhibits hydroxylation of the HIF-1α $P_{564}$ residue or a homologous proline in another HIFα isoform. In another specific embodiment, the agent inhibits hydroxylation of the HIF-1α $P_{402}$ residue or a homologous proline in another HIFα isoform. In yet another embodiment, the compound may additionally inhibit hydroxylation of HIFα asparagine residues. In one specific embodiment, the agent inhibits hydroxylation of the HIF-1α $N_{803}$ residue or a homologous asparagine residue in another HIFα isoform.

[0033] In certain embodiments, compounds used in the methods of the invention are selected from a compound of the formula (I)




(I)

wherein

A is 1,2-arylidene, 1,3-arylidene, 1,4-arylidene; or ($C_1$-$C_4$)-alkylene, optionally substituted by one or two halogen, cyano, nitro, trifluoromethyl, ($C_1$-$C_6$)-alkyl, ($C_1$-$C_6$)-hydroxyalkyl, ($C_1$-$C_6$)-alkoxy, -O-$[CH_2]_x$-$C_fH_{(2f+1-g)}Hal_g$, ($C_1$-$C_6$)-fluoroalkoxy, ($C_1$-$C_8$)-fluoroalkenyloxy, ($C_1$-$C_8$)-fluoroalkynyloxy, -$OCF_2Cl$, -O-$CF_2$-CHFCl; ($C_1$-$C_6$)-alkylmercapto, ($C_1$-$C_6$)-alkylsulfinyl, ($C_1$-$C_6$)-alkylsulfonyl, ($C_1$-$C_6$)-alkylcarbonyl, ($C_1$-$C_6$)-alkoxycarbonyl, carbamoyl, N-($C_1$-$C_4$)-alkylcarbamoyl, N,N-di-($C_1$-$C_4$)-alkylcarbamoyl, ($C_1$-$C_6$)-alkylcarbonyloxy, ($C_3$-$C_8$)-cycloalkyl, phenyl, benzyl, phenoxy, benzyloxy, anilino, N-methylanilino, phenylmercapto, phenylsulfonyl, phenylsulfinyl, sulfamoyl, N-($C_1$-$C_4$)-alkylsulfamoyl, N,N-di-($C_1$-$C_4$)-alkylsulfamoyl; or by a substituted ($C_6$-$C_{12}$)-aryloxy, ($C_7$-$C_{11}$)-aralkyloxy, ($C_6$-$C_{12}$)-aryl, ($C_7$-$C_{11}$)-aralkyl radical, which carries in the aryl moiety one to five identical or different substituents selected from halogen, cyano, nitro, trifluoromethyl, ($C_1$-$C_6$)-alkyl, ($C_1$-$C_6$)-alkoxy, -O-$[CH_2]_x$-$C_fH_{(2f+1-g)}Hal_g$, -$OCF_2Cl$, -O-$CF_2$-CHFCl, ($C_1$-$C_6$)-alkylmercapto, ($C_1$-$C_6$)-alkylsulfinyl, ($C_1$-$C_6$)-alkylsulfonyl, ($C_1$-$C_6$)-alkylcarbonyl, ($C_1$-$C_6$)-alkoxycarbonyl, carbamoyl, N-($C_1$-$C_4$)-alkylcarbamoyl, N,N-di-($C_1$-$C_4$)-alkylcarbamoyl, ($C_1$-

$C_6$)-alkylcarbonyloxy, ($C_3$-$C_8$)-cycloalkyl, sulfamoyl, N-($C_1$-$C_4$)-alkylsulfamoyl, N,N-di-($C_1$-$C_4$)-alkylsulfamoyl; or wherein A is -$CR^5R^6$ and $R^5$ and $R^6$ are each independently selected from hydrogen, ($C_1$-$C_6$)-alkyl, ($C_3$-$C_7$)-cycloalkyl, aryl, or a substituent of the $\alpha$-carbon atom of an $\alpha$-amino acid, wherein the amino acid is a natural L-amino acid or its D-isomer.

B is -$CO_2H$, -$NH_2$, -$NHSO_2CF_3$, tetrazolyl, imidazolyl, 3-hydroxyisoxazolyl, -CONHCOR"', -CONHSOR"', $CONHSO_2R$"', where R"' is aryl, heteroaryl, ($C_3$-$C_7$)-cycloalkyl, or ($C_1$-$C_4$)-alkyl, optionally monosubstituted by ($C_6$-$C_{12}$)-aryl, heteroaryl, OH, SH, ($C_1$-$C_4$)-alkyl, ($C_1$-$C_4$)-alkoxy, ($C_1$-$C_4$)-thioalkyl, ($C_1$-$C_4$)-sulfinyl, ($C_1$-$C_4$)-sulfonyl, $CF_3$, Cl, Br, F, I, NO2, -COOH, ($C_2$-$C_5$)-alkoxycarbonyl; $NH_2$, mono-($C_1$-$C_4$-alkyl)-amino, di-($C_1$-$C_4$-alkyl)-amino, or ($C_1$-$C_4$)-perfluoroalkyl; or wherein B is a $CO_2$-G carboxyl radical, where G is a radical of an alcohol G-OH in which G is selected from ($C_1$-$C_{20}$)-alkyl radical, ($C_3$-$C_8$) cycloalkyl radical, ($C_2$-$C_{20}$)-alkenyl radical, ($C_3$-$C_8$)-cycloalkenyl radical, retinyl radical, ($C_2$-$C_{20}$)-alkynyl radical, ($C_4$-$C_{20}$)-alkenynyl radical, where the alkenyl, cycloalkenyl, alkynyl, and alkenynyl radicals contain one or more multiple bonds; ($C_6$-$C_{16}$)-carbocyclic aryl radical, ($C_7$-$C_{16}$)-carbocyclic aralkyl radical, heteroaryl radical, or heteroaralkyl radical, wherein a heteroaryl radical or heteroaryl moiety of a heteroaralkyl radical contains 5 or 6 ring atoms; and wherein radicals defined for G are substituted by one or more hydroxyl, halogen, cyano, trifluoromethyl, nitro, carboxyl, ($C_1$-$C_{12}$)-alkyl, ($C_3$-$C_8$)-cycloalkyl, ($C_5$-$C_8$)-cycloalkenyl, ($C_6$-$C_{12}$)-aryl, ($C_7$-$C_{16}$)-aralkyl, ($C_2$-$C_{12}$)-alkenyl, ($C_2$-$C_{12}$)-alkynyl, ($C_1$-$C_{12}$)-alkoxy, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)-alkyl, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)-alkoxy, ($C_6$-$C_{12}$)-aryloxy, ($C_7$-$C_{16}$)-aralkyloxy, ($C_1$-$C_8$)-hydroxyalkyl, -O-$[CH_2]_x$-$C_fH_{(2f+1-g)}$-$F_g$, -$OCF_2Cl$, -$OCF_2$-CHFCl, ($C_1$-$C_{12}$)-alkylcarbonyl, ($C_3$-$C_8$)-cycloalkylcarbonyl, ($C_6$-$C_{12}$)-arylcarbonyl, ($C_7$-$C_{16}$)-aralkylcarbonyl, cinnamoyl, ($C_2$-$C_{12}$)-alkenylcarbonyl, ($C_2$-$C_{12}$)-alkynylcarbonyl, ($C_1$-$C_{12}$)-alkoxycarbonyl, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)-alkoxycarbonyl, ($C_6$-$C_{12}$)-aryloxycarbonyl, ($C_7$-$C_{16}$)-aralkoxycarbonyl, ($C_3$-$C_8$)-cycloalkoxycarbonyl, ($C_2$-$C_{12}$)-alkenyloxycarbonyl, ($C_2$-$C_{12}$)-alkynyloxycarbonyl, acyloxy, ($C_1$-$C_{12}$)-alkoxycarbonyloxy, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)-alkoxycarbonyloxy, ($C_6$-$C_{12}$)-aryloxycarbonyloxy, ($C_7$-$C_{16}$) aralkyloxycarbonyloxy, ($C_3$-$C_8$)-cycloalkoxycarbonyloxy, ($C_2$-$C_{12}$)-alkenyloxycarbonyloxy, ($C_2$-$C_{12}$)-alkynyloxycarbonyloxy, carbamoyl, N-($C_1$-$C_{12}$)-alkylcarbamoyl, N.N-di($C_1$-$C_{12}$)-alkylcarbamoyl, N-($C_3$-$C_8$)-cycloalkyl-carbamoyl, N-($C_6$-$C_{16}$)-arylcarbamoyl, N-($C_7$-$C_{16}$)-aralkylcarbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_6$-$C_{16}$)-arylcarbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_7$-$C_{16}$)-aralkylcarbamoyl, N-(($C_1$-$C_{10}$)-alkoxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-(($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_{10}$)alkyl)-carbamoyl, N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-(($C_1$-$C_{10}$)-alkoxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-(($C_6$-$C_{16}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, carbamoyloxy, N-($C_1$-$C_{12}$)-alkylcarbamoyloxy, N.N-di-($C_1$-$C_{12}$)-alkylcarbamoyloxy, N-($C_3$-$C_8$)-cycloalkylcarbamoyloxy,

N-($C_6$-$C_{12}$)-arylcarbamoyloxy, N-($C_7$-$C_{16}$)-aralkylcarbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-($C_6$-$C_{12}$)-arylcarbamoyloxy, N($C_1$-$C_{10}$)-alkyl-N-($C_7$-$C_{16}$)-aralkylcarbamoyloxy, N-(($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, N-(($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-(($C_1$-$C_{10}$)-alkoxy-($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-(($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, amino, ($C_1$-$C_{12}$)-alkylamino, di-($C_1$-$C_{12}$)-alkylamino, ($C_3$-$C_8$)-cycloalkylamino, ($C_2$-$C_{12}$)-alkenylamino, ($C_2$-$C_{12}$)-alkynylamino, N-($C_6$-$C_{12}$)-arylamino, N-(C-$C_{11}$)-aralkylamino, N-alkyl-aralkylamino, N-alkyl-arylamino, ($C_1$-$C_{12}$)-alkoxyamino, ($C_1$-$C_{12}$)-alkoxy-N-($C_1$-$C_{10}$)-alkylamino, ($C_1$-$C_{12}$)-alkylcarbonylamino, ($C_3$-$C_8$)-cycloalkylcarbonylamino, ($C_6$-$C_{12}$) arylcarbonylamino, ($C_7$-$C_{16}$)-aralkylcarbonylamino, ($C_1$-$C_{12}$)-alkylcarbonyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_3$-$C_8$)-cycloalkylcarbonyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_6$-$C_{12}$)-arylcarbonyl-N-($C_1$-$C_{10}$)alkylamino, ($C_7$-$C_{11}$)-aralkylcarbonyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_1$-$C_{12}$)-alkylcarbonylamino-($C_1$-$C_8$)-alkyl, ($C_3$-$C_8$)-cycloalkylcarbonylamino-($C_1$-$C_8$)alkyl, ($C_6$-$C_{12}$)-arylcarbonylamino-($C_1$-$C_8$)-alkyl, ($C_7$-$C_{12}$)-aralkylcarbonylamino($C_1$-$C_8$)-alkyl, amino-($C_1$-$C_{10}$)-alkyl, N-($C_1$-$C_{10}$) alkylamino-($C_1$-$C_{10}$)-alkyl, N.N-di-($C_1$-$C_{10}$)-alkylamino-($C_1$-$C_{10}$)-alkyl, ($C_3$-$C_8$)cycloalkylamino-($C_1$-$C_{10}$)-alkyl, ($C_1$-$C_{12}$)-alkylmercapto, ($C_1$-$C_{12}$)-alkylsulfinyl, ($C_1$-$C_{12}$)-alkylsulfonyl, ($C_6$-$C_{16}$)-arylmercapto, ($C_6$-$C_{16}$)-arylsulfinyl, ($C_6$-$C_{12}$)-arylsulfonyl, ($C_7$-$C_{16}$)-aralkylmercapto, ($C_7$-$C_{16}$)-aralkylsulfinyl, ($C_7$-$C_{16}$)-aralkylsulfonyl, sulfamoyl, N-($C_1$-$C_{10}$)-alkylsulfamoyl, N.N-di($C_1$-$C_{10}$)-alkylsulfamoyl, ($C_3$-$C_8$)-cycloalkylsulfamoyl, N-($C_6$-$C_{12}$)-alkylsulfamoyl, N-($C_7$-$C_{16}$)-aralkylsulfamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_6$-$C_{12}$)-arylsulfamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_7$-$C_{16}$)-aralkylsulfamoyl, ($C_1$-$C_{10}$)-alkylsulfonamido, N-(($C_1$-$C_{10}$)-alkyl)-($C_1$-$C_{10}$)-alkylsulfonamido, ($C_7$-$C_{16}$)-aralkylsulfonamido, or N-(($C_1$-$C_{10}$)-alkyl-($C_7$-$C_{16}$)-aralkylsulfonamido; wherein radicals which are aryl or contain an aryl moiety, may be substituted on the aryl by one to five identical or different hydroxyl, halogen, cyano, trifluoromethyl, nitro, carboxyl, ($C_1$-$C_{12}$)-alkyl, ($C_3$-$C_8$)-cycloalkyl, ($C_6$-$C_{12}$)-aryl, ($C_7$-$C_{16}$)-aralkyl, ($C_1$-$C_{12}$)-alkoxy, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)alkyl, ($C_1$-$C_{12}$)-alkoxy-($C_1$ $C_{12}$)alkoxy, ($C_6$-$C_{12}$)-aryloxy, ($C_7$-$C_{16}$)-aralkyloxy, ($C_1$-$C_8$)-hydroxyalkyl, ($C_1$-$C_{12}$)-alkylcarbonyl, ($C_3$-$C_8$)-cycloalkyl-carbonyl, ($C_6$-$C_{12}$)-arylcarbonyl, ($C_7$-$C_{16}$) aralkylcarbonyl, ($C_1$-$C_{12}$)-alkoxycarbonyl, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)-alkoxycarbonyl, ($C_6$-$C_{12}$)-aryloxycarbonyl, ($C_7$-$C_{16}$)-aralkoxycarbonyl, ($C_3$-$C_8$)-cycloalkoxycarbonyl, ($C_2$-$C_{12}$)-alkenyloxycarbonyl, ($C_2$-$C_{12}$)-alkynyloxycarbonyl, ($C_1$-$C_{12}$)-alkylcarbonyloxy, ($C_3$-$C_8$)-cycloalkylcarbonyloxy, ($C_6$-$C_{12}$)-arylcarbonyloxy, ($C_7$-$C_{16}$)-aralkylcarbonyloxy, cinnamoyloxy, ($C_2$-$C_{12}$)-alkenylcarbonyloxy, ($C_2$-$C_{12}$)-alkynylcarbonyloxy, ($C_1$-$C_{12}$)-alkoxycarbonyloxy, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)-alkoxycarbonyloxy, ($C_6$-$C_{12}$)-aryloxycarbonyloxy, ($C_7$-$C_{16}$)-aralkyloxycarbonyloxy, ($C_3$-$C_8$)-cycloalkoxycarbonyloxy, ($C_2$-$C_{12}$)-alkenyloxycarbonyloxy, ($C_2$-$C_{12}$)-alkynyloxycarbonyloxy, carbamoyl, N-($C_1$-$C_{12}$)-alkylcarbamoyl, N.N-di-($C_1$-$C_{12}$)-alkylcarbamoyl, N-($C_3$-$C_8$)-cycloalkylcarbamoyl, N-($C_6$-

$C_{12}$)-arylcarbamoyl, N-($C_7$-$C_{16}$)-aralkylcarbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_6$-$C_{12}$)-arylcarbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_7$-$C_{16}$)-aralkylcarbamoyl, N-(($C_1$-$C_{10}$)-alkoxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-(($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-(($C_1$-$C_{10}$)-alkoxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-(($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, carbamoyloxy, N-($C_1$-$C_{12}$)-alkylcarbamoyloxy, N.N-di-($C_1$-$C_{12}$)-alkylcarbamoyloxy, N-($C_3$-$C_8$)-cycloalkylcarbamoyloxy, N-($C_6$-$C_{12}$)-arylcarbamoyloxy, N-($C_7$-$C_{16}$)-aralkylcarbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-($C_6$-$C_{12}$)-arylcarbamoyloxy, N($C_1$-$C_{10}$)-alkyl-N-($C_7$-$C_{16}$)-aralkylcarbamoyloxy, N-(($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, N-(($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-(($C_1$-$C_{10}$)-alkoxy-($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-(($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, amino, ($C_1$-$C_{12}$)-alkylamino, di-($C_1$-$C_{12}$)-alkylamino, ($C_3$-$C_8$)-cycloalkylamino, ($C_3$-$C_{12}$)-alkenylamino, ($C_3$-$C_{12}$)-alkynylamino, N-($C_6$-$C_{12}$)-arylamino, N-($C_7$-$C_{11}$)-aralkylamino, N-alkylaralkylamino, N-alkyl-arylamino, ($C_1$-$C_{12}$)-alkoxyamino, ($C_1$-$C_{12}$)-alkoxy-N-($C_1$-$C_{10}$)-alkylamino, ($C_1$-$C_{12}$)-alkylcarbonylamino, ($C_3$-$C_8$)-cycloalkylcarbonylamino, ($C_6$-$C_{12}$)-arylcarbonylamino, ($C_7$-$C_{16}$)-alkylcarbonylamino, ($C_1$-$C_{12}$)-alkylcarbonyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_3$-$C_8$)-cycloalkylcarbonyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_6$-$C_{12}$)-arylcarbonyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_7$-$C_{11}$)-aralkylcarbonyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_1$-$C_{12}$)-alkylcarbonylamino-($C_1$-$C_8$)-alkyl, ($C_3$-$C_8$)-cycloalkylcarbonylamino-($C_1$-$C_8$)-alkyl, ($C_6$-$C_{12}$)-arylcarbonylamino-($C_1$-$C_8$)-alkyl, ($C_7$-$C_{16}$)-aralkylcarbonylamino-($C_1$-$C_8$)-alkyl, amino-($C_1$-$C_{10}$)-alkyl, N-($C_1$-$C_{10}$)-alkylamino-($C_1$-$C_{10}$)alkyl, N.N-di-($C_1$-$C_{10}$)-alkylamino-($C_1$-$C_{10}$)-alkyl, ($C_3$-$C_8$)-cycloalkylamino-($C_1$-$C_{10}$)-alkyl, ($C_1$-$C_{12}$)-alkylmercapto, ($C_1$-$C_{12}$)-alkylsulfinyl, ($C_1$-$C_{12}$)-alkylsulfonyl, ($C_6$-$C_{12}$)-arylmercapto, ($C_6$-$C_{12}$)-arylsulfinyl, ($C_6$-$C_{12}$)-arylsulfonyl, ($C_7$-$C_{16}$)-aralkylmercapto, ($C_7$-$C_{16}$)-aralkylsulfinyl, or ($C_7$-$C_{16}$)-aralkylsulfonyl;

X is O or S;

Q is O, S, NR', or a bond;

where, if Q is a bond, $R^4$ is halogen, nitrile, or trifluoromethyl;

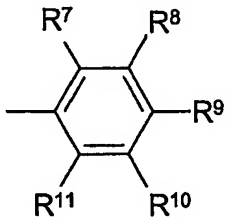
or where, if Q is O, S, or NR', $R^4$ is hydrogen, ($C_1$-$C_{10}$)-alkyl radical, ($C_2$-$C_{10}$)-alkenyl radical, ($C_2$-$C_{10}$)-alkynyl radical, wherein alkenyl or alkynyl radical contains one or two C-C multiple bonds; unsubstituted fluoroalkyl radical of the formula -$[CH_2]_x$-$C_fH_{(2f+1-g)}$-$F_g$, ($C_1$-$C_8$)-alkoxy-

($C_1$-$C_6$)-alkyl radical, ($C_1$-$C_6$)-alkoxy-($C_1$-$C_4$)-alkoxy-($C_1$-$C_4$)-alkyl radical, aryl radical, heteroaryl radical, ($C_7$-$C_{11}$)-aralkyl radical, or a radical of the formula Z

$$-[CH_2]_v-[O]_w-[CH_2]_t-E \qquad (Z)$$

where

E is a heteroaryl radical, a ($C_3$-$C_8$)-cycloalkyl radical, or a phenyl radical of the formula F




(F)

v is 0-6,

w is 0 or 1,

t is 0-3, and

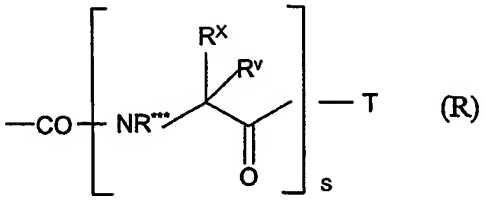
$R^7$, $R^8$, $R^9$, $R^{10}$, and $R^{11}$ are identical or different and are hydrogen, halogen, cyano, nitro, trifluoromethyl, ($C_1$-$C_6$)-alkyl, ($C_3$-$C_8$)-cycloalkyl, ($C_1$-$C_6$)-alkoxy, -O-$[CH_2]_x$-$C_fH_{(2f+1-g)}$-$F_g$, -$OCF_2$-Cl, -O-$CF_2$-CHFCl, ($C_1$-$C_6$)-alkylmercapto, ($C_1$-$C_6$)-hydroxyalkyl, ($C_1$-$C_6$)-alkoxy-($C_1$-$C_6$)-alkoxy, ($C_1$-$C_6$)-alkoxy-($C_1$-$C_6$)-alkyl, ($C_1$-$C_6$)-alkylsulfinyl, ($C_1$-$C_6$)-alkylsulfonyl, ($C_1$-$C_6$)-alkylcarbonyl, ($C_1$-$C_8$)-alkoxycarbonyl, carbamoyl, N-($C_1$-$C_8$)-alkylcarbamoyl, N,N-di-($C_1$-$C_8$)-alkylcarbamoyl, or ($C_7$-$C_{11}$)-aralkylcarbamoyl, optionally substituted by fluorine, chlorine, bromine, trifluoromethyl, ($C_1$-$C_6$)-alkoxy, N-($C_3$-$C_8$)-cycloalkylcarbamoyl, N-($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_4$)-alkylcarbamoyl, ($C_1$-$C_6$)-alkylcarbonyloxy, phenyl, benzyl, phenoxy, benzyloxy, $NR^YR^Z$ wherein $R^y$ and $R^z$ are independently selected from hydrogen, ($C_1$-$C_{12}$)-alkyl, ($C_1$-$C_8$)-alkoxy-($C_1$-$C_8$)-alkyl, ($C_7$-$C_{12}$)-aralkoxy-($C_1$-$C_8$)-alkyl, ($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_8$)-alkyl, ($C_3$-$C_{10}$)-cycloalkyl, ($C_3$-$C_{12}$)-alkenyl, ($C_3$-$C_{12}$)-alkynyl, ($C_6$-$C_{12}$)-aryl, ($C_7$-$C_{11}$)-aralkyl, ($C_1$-$C_{12}$)-alkoxy, ($C_7$-$C_{12}$)aralkoxy, ($C_1$-$C_{12}$)-alkylcarbonyl, ($C_3$-$C_8$)-cycloalkylcarbonyl, ($C_6$-$C_{12}$) arylcarbonyl, ($C_7$-$C_{16}$)-aralkylcarbonyl; or further wherein $R^y$ and $R^z$ together are -[CH2 ]$_h$ , in which a $CH_2$ group can be replaced by O, S, N-($C_1$-$C_4$)-alkylcarbonylimino, or N-($C_1$-$C_4$)-alkoxycarbonylimino; phenylmercapto, phenylsulfonyl, phenylsulfinyl, sulfamoyl, N-($C_1$-$C_8$)-alkylsulfamoyl, or N, N-di-($C_1$-$C_8$)-alkylsulfamoyl; or alternatively $R^7$ and $R^8$, $R^8$ and $R^9$, $R^9$ and $R^{10}$, or $R^{10}$ and $R^{11}$, together are a chain selected from -$[CH_2]_n$- or -CH=CH-CH=CH-, where a $CH_2$ group of the chain is optionally replaced by O, S, SO, $SO_2$, or $NR^Y$; and n is 3, 4, or 5; and if E is a heteroaryl radical, said radical can carry 1-3 substituents selected from those defined for $R^7$-$R^{11}$, or if E is a cycloalkyl radical, the radical can carry one substituent selected from those defined for $R^7$-$R^{11}$;

or where, if Q is NR', $R^4$ is alternatively R", where R' and R" are identical or different and are hydrogen, ($C_6$-$C_{12}$)-aryl, ($C_7$-$C_{11}$)-aralkyl, ($C_1$-$C_8$)-alkyl, ($C_1$-$C_8$)-alkoxy-($C_1$-$C_8$)-alkyl, ($C_7$-$C_{12}$)-aralkoxy-($C_1$-$C_8$)-alkyl, ($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_8$)-alkyl, ($C_1$-$C_{10}$)-alkylcarbonyl, optionally substituted ($C_7$-$C_{16}$)-aralkylcarbonyl, or optionally substituted $C_6$-$C_{12}$)-arylcarbonyl; or R' and R" together are -$[CH_2]_h$, in which a $CH_2$ group can be replaced by O, S, N-acylimino, or N-($C_1$-$C_{10}$)-alkoxycarbonylimino, and h is 3 to 7.

Y is N or $CR^3$;

$R^1$, $R^2$ and $R^3$ are identical or different and are hydrogen, hydroxyl, halogen, cyano, trifluoromethyl, nitro, carboxyl, ($C_1$-$C_{20}$)-alkyl, ($C_3$-$C_8$)-cycloalkyl, ($C_3$-$C_8$)cycloalkyl-($C_1$-$C_{12}$)-alkyl, ($C_3$-$C_8$)-cycloalkoxy, ($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_{12}$)-alkoxy, ($C_3$-$C_8$)-cycloalkyloxy-($C_1$-$C_{12}$)-alkyl, ($C_3$-$C_8$)-cycloalkyloxy-($C_1$-$C_{12}$)-alkoxy, ($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_8$)-alkyl-($C_1$-$C_6$)-alkoxy, ($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_8$)-alkoxy-($C_1$-$C_6$)-alkyl, ($C_3$-$C_8$)-cycloalkyloxy-($C_1$-$C_8$)-alkoxy-($C_1$-$C_6$)-alkyl, ($C_3$-$C_8$)-cycloalkoxy-($C_1$-$C_8$)-alkoxy-($C_1$-$C_8$)-alkoxy, ($C_6$-$C_{12}$)-aryl, ($C_7$-$C_{16}$)-aralkyl, ($C_7$-$C_{16}$)-aralkenyl, ($C_7$-$C_{16}$)-aralkynyl, ($C_2$-$C_{20}$)-alkenyl, ($C_2$-$C_{20}$)-alkynyl, ($C_1$-$C_{20}$)-alkoxy, ($C_2$-$C_{20}$)-alkenyloxy, ($C_2$-$C_{20}$)-alkynyloxy, retinyloxy, ($C_1$-$C_{20}$)-alkoxy-($C_1$-$C_{12}$)-alkyl, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)-alkoxy, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_8$)-alkoxy-($C_1$-$C_8$)-alkyl, ($C_6$-$C_{12}$)-aryloxy, ($C_7$-$C_{16}$)-aralkyloxy, ($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_6$)-alkoxy, ($C_7$-$C_{16}$)-aralkoxy-($C_1$-$C_6$)-alkoxy, ($C_1$-$C_{16}$)-hydroxyalkyl, ($C_6$-$C_{16}$)-aryloxy-($C_1$-$C_8$)-alkyl, ($C_7$-$C_{16}$)-aralkoxy-($C_1$-$C_8$)-alkyl, ($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_8$)-alkoxy-($C_1$-$C_6$)-alkyl, ($C_7$-$C_{12}$)-aralkyloxy-($C_1$-$C_8$)-alkoxy-($C_1$-$C_6$)-alkyl, ($C_2$-$C_{20}$)-alkenyloxy-($C_1$-$C_6$)-alkyl, ($C_2$-$C_{20}$)-alkynyloxy-($C_1$-$C_6$)-alkyl, retinyloxy-($C_1$-$C_6$)-alkyl, -O-$[CH_2]_xCfH_{(2f+1-g)}F_g$, -$OCF_2Cl$, -$OCF_2$-CHFCl, ($C_1$-$C_{20}$)-alkylcarbonyl, ($C_3$-$C_8$)-cycloalkylcarbonyl, ($C_6$-$C_{12}$)-arylcarbonyl, ($C_7$-$C_{16}$)-aralkylcarbonyl, cinnamoyl, ($C_2$-$C_{20}$)-alkenylcarbonyl, ($C_2$-$C_{20}$)-alkynylcarbonyl, ($C_1$-$C_{20}$)-alkoxycarbonyl, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)-alkoxycarbonyl, ($C_6$-$C_{12}$)-aryloxycarbonyl, ($C_7$-$C_{16}$)-aralkoxycarbonyl, ($C_3$-$C_8$)-cycloalkoxycarbonyl, ($C_2$-$C_{20}$)-alkenyloxycarbonyl, retinyloxycarbonyl, ($C_2$-$C_{20}$)-alkynyloxycarbonyl, ($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_6$)-alkoxycarbonyl, ($C_7$-$C_{16}$)-aralkoxy-($C_1$-$C_6$)-alkoxycarbonyl, ($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_6$)-alkoxycarbonyl, ($C_3$-$C_8$)-cycloalkoxy-($C_1$-$C_6$)-alkoxycarbonyl, ($C_1$-$C_{12}$)-alkylcarbonyloxy, ($C_3$-$C_8$)-cycloalkylcarbonyloxy, ($C_6$-$C_{12}$)-arylcarbonyloxy, ($C_7$-$C_{16}$)-aralkylcarbonyloxy, cinnamoyloxy, ($C_2$-$C_{12}$)-alkenylcarbonyloxy, ($C_2$-$C_{12}$)-alkynylcarbonyloxy, ($C_1$-$C_{12}$)-alkoxycarbonyloxy, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)-alkoxycarbonyloxy, ($C_6$-$C_{12}$)-aryloxycarbonyloxy, ($C_7$-$C_{16}$)-aralkyloxycarbonyloxy, ($C_3$-$C_8$)-cycloalkoxycarbonyloxy, ($C_2$-$C_{12}$)-alkenyloxycarbonyloxy, ($C_2$-$C_{12}$)-alkynyloxycarbonyloxy, carbamoyl, N-($C_1$-$C_{12}$)-alkylcarbamoyl, N,N-di-($C_1$-$C_{12}$)-alkylcarbamoyl, N-($C_3$-$C_8$)-cycloalkylcarbamoyl, N,N-dicyclo-($C_3$-$C_8$)-alkylcarbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_3$-$C_8$)-cycloalkylcarbamoyl, N-(($C_3$-

$C_8$)-cycloalkyl-($C_1$-$C_6$)-alkyl)-carbamoyl, N-($C_1$-$C_6$)-alkyl-N-(($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_6$)-alkyl)-carbamoyl, N-(+)-dehydroabietylcarbamoyl, N-($C_1$-$C_6$)-alkyl-N-(+)-dehydroabietylcarbamoyl, N-($C_6$-$C_{12}$)-arylcarbamoyl, N-($C_7$-$C_{16}$)-aralkylcarbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_6$-$C_{16}$)-arylcarbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_7$-$C_{16}$)-aralkylcarbamoyl, N-(($C_1$-$C_{18}$)-alkoxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-(($C_6$-$C_{16}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-(($C_1$-$C_{10}$)-alkoxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-(($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl; $CON(CH_2)_h$, in which a $CH_2$ group can be replaced by O, S, N-($C_1$-$C_8$)-alkylimino, N-($C_3$-$C_8$)-cycloalkylimino, N-($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_4$)-alkylimino, N-($C_6$-$C_{12}$)-arylimino, N-($C_7$-$C_{16}$)-aralkylimino, N-($C_1$-$C_4$)-alkoxy-($C_1$-$C_6$)-alkylimino, and h is from 3 to 7; a carbamoyl radical of the formula R




(R)

in which

$R^x$ and $R^v$ are each independently selected from hydrogen, ($C_1$-$C_6$)-alkyl, ($C_3$-$C_7$)-cycloalkyl, aryl, or the substituent of an $\alpha$-carbon of an $\alpha$-amino acid, to which the L- and D-amino acids belong,

s is 1-5,

T is OH, or NR*R**, and R*, R** and R*** are identical or different and are selected from hydrogen, ($C_6$-$C_{12}$)-aryl, ($C_7$-$C_{11}$)-aralkyl, ($C_1$-$C_8$)-alkyl, ($C_3$-$C_8$)-cycloalkyl, (+)-dehydroabietyl, ($C_1$-$C_8$)-alkoxy-($C_1$-$C_8$)-alkyl, ($C_7$-$C_{12}$)-aralkoxy-($C_1$-$C_8$)-alkyl, ($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_8$)-alkyl, ($C_1$-$C_{10}$)-alkanoyl, optionally substituted ($C_7$-$C_{16}$)-aralkanoyl, optionally substituted ($C_6$-$C_{12}$)-aroyl; or R* and R** together are -$[CH_2]_h$, in which a $CH_2$ group can be replaced by O, S, SO, $SO_2$, N-acylamino, N-($C_1$-$C_{10}$)-alkoxycarbonylimino, N-($C_1$-$C_8$)-alkylimino, N-($C_3$-$C_8$)-cycloalkylimino, N-($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_4$)-alkylimino, N-($C_6$-$C_{12}$)-arylimino, N-($C_7$-$C_{16}$)-aralkylimino, N-($C_1$-$C_4$)-alkoxy-($C_1$-$C_6$)-alkylimino, and h is from 3 to 7;

carbamoyloxy, N-($C_1$-$C_{12}$)-alkylcarbamoyloxy, N,N-di-($C_1$-$C_{12}$)-alkylcarbamoyloxy, N-($C_3$-$C_8$)-cycloalkylcarbamoyloxy, N-($C_6$-$C_{12}$)-arylcarbamoyloxy, N-($C_7$-$c_{16}$)-aralkylcarbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-($C_6$-$C_{12}$)-arylcarbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-($C_7$-$C_{16}$)-aralkylcarbamoyloxy, N-(($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, N-(($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-(($C_1$-$C_{10}$)-alkoxy-($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-(($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)-

carbamoyloxyamino, ($C_1$-$C_{12}$)-alkylamino, di-($C_1$-$C_{12}$)-alkylamino, ($C_3$-$C_8$)-cycloalkylamino, ($C_3$-$C_{12}$)-alkenylamino, ($C_3$-$C_{12}$)-alkynylamino, N-($C_6$-$C_{12}$)-arylamino, N-($C_7$-$C_{11}$)-aralkylamino, N-alkyl-aralkylamino, N-alkyl-arylamino, ($C_1$-$C_{12}$)-alkoxyamino, ($C_1$-$C_{12}$)-alkoxy-N-($C_1$-$C_{10}$)-alkylamino, ($C_1$-$C_{12}$)-alkanoylamino, ($C_3$-$C_8$)-cycloalkanoylamino, ($C_6$-$C_{12}$)-aroylamino, ($C_7$-$C_{16}$)-aralkanoylamino, ($C_1$-$C_{12}$)-alkanoyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_3$-$C_8$)-cycloalkanoyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_6$-$C_{12}$)-aroyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_7$-$C_{11}$)-aralkanoyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_1$-$C_{12}$)-alkanoylamino-($C_1$-$C_8$)-alkyl, ($C_3$-$C_8$)-cycloalkanoylamino-($C_1$-$C_8$)-alkyl, ($C_6$-$C_{12}$)-aroylamino-($C_1$-$C_8$)-alkyl, ($C_7$-$C_{16}$)-aralkanoylamino-($C_1$-$C_8$)-alkyl, amino-($C_1$-$C_{10}$)-alkyl, N-($C_1$-$C_{10}$)-alkylamino-($C_1$-$C_{10}$)-alkyl, N,N-di($C_1$-$C_{10}$)-alkylamino-($C_1$-$C_{10}$)-alkyl, ($C_3$-$C_8$)-cycloalkylamino($C_1$-$C_{10}$)-alkyl, ($C_1$-$C_{20}$)-alkylmercapto, ($C_1$-$C_{20}$)-alkylsulfinyl, ($C_1$-$C_{20}$)-alkylsulfonyl, ($C_6$-$C_{12}$)-arylmercapto, ($C_6$-$C_{12}$)-arylsulfinyl, ($C_6$-$C_{12}$)-arylsulfonyl, ($C_7$-$C_{16}$)-aralkylmercapto, ($C_7$-$C_{16}$)-aralkylsulfinyl, ($C_7$-$C_{16}$)-aralkylsulfonyl, ($C_1$-$C_{12}$)-alkylmercapto-($C_1$-$C_6$)-alkyl, ($C_1$-$C_{12}$)-alkylsulfinyl-($C_1$-$C_6$)-alkyl, ($C_1$-$C_{12}$)-alkylsulfonyl-($C_1$-$C_6$)-alkyl, ($C_6$-$C_{12}$)-arylmercapto-($C_1$-$C_6$)-alkyl, ($C_6$-$C_{12}$)-arylsulfinyl-($C_1$-$C_6$)-alkyl, ($C_6$-$C_{12}$)-arylsulfonyl-($C_1$-$C_6$)-alkyl, ($C_7$-$C_{16}$)-aralkylmercapto-($C_1$-$C_6$)-alkyl, ($C_7$-$C_{16}$)-aralkylsulfinyl-($C_1$-$C_6$)-alkyl, ($C_7$-$C_{16}$)-aralkylsulfonyl-($C_1$-$C_6$)-alkyl, sulfamoyl, N-($C_1$-$C_{10}$)-alkylsulfamoyl, N,N-di-($C_1$-$C_{10}$)-alkylsulfamoyl, ($C_3$-$C_8$)-cycloalkylsulfamoyl, N-($C_6$-$C_{12}$)-arylsulfamoyl, N-($C_7$-$C_{16}$)-aralkylsulfamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_6$-$C_{12}$)-arylsulfamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_7$-$C_{16}$)-aralkylsulfamoyl, ($C_1$-$C_{10}$)-alkylsulfonamido, N-(($C_1$-$C_{10}$)-alkyl)-($C_1$-$C_{10}$)-alkylsulfonamido, ($C_7$-$C_{16}$)-aralkylsulfonamido, and N-(($C_1$-$C_{10}$)-alkyl-($C_7$-$C_{16}$)-aralkylsulfonamido; where an aryl radical may be substituted by 1 to 5 substituents selected from hydroxyl, halogen, cyano, trifluoromethyl, nitro, carboxyl, ($C_2$-$C_{16}$)-alkyl, ($C_3$-$C_8$)-cycloalkyl, ($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_{12}$)-alkyl, ($C_3$-$C_8$)-cycloalkoxy, ($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_{12}$)-alkoxy, ($C_3$-$C_8$)-cycloalkyloxy-($C_1$-$C_{12}$)-alkyl, ($C_3$-$C_8$)-cycloalkyloxy-($C_1$-$C_{12}$)-alkoxy, ($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_8$)-alkyl-($C_1$-$C_6$)-alkoxy, ($C_3$-$C_8$)-cycloalkyl($C_1$-$C_8$)-alkoxy-($C_1$-$C_6$)-alkyl, ($C_3$-$C_8$)-cycloalkyloxy-($C_1$-$C_8$)-alkoxy-($C_1$-$C_6$)-alkyl, ($C_3$-$C_8$)-cycloalkoxy-($C_1$-$C_8$)-alkoxy-($C_1$-$C_8$)-alkoxy, ($C_6$-$C_{12}$)-aryl, ($C_7$-$C_{16}$)-aralkyl, ($C_2$-$C_{16}$)-alkenyl, ($C_2$-$C_{12}$)-alkynyl, ($C_1$-$C_{16}$)-alkoxy, ($C_1$-$C_{16}$)-alkenyloxy, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)-alkyl, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)-alkoxy, ($C_1$-$C_{12}$)-alkoxy($C_1$-$C_8$)-alkoxy-($C_1$-$C_8$)-alkyl, ($C_6$-$C_{12}$)-aryloxy, ($C_7$-$C_{16}$)-aralkyloxy, ($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_6$)-alkoxy, ($C_7$-$C_{16}$)-aralkoxy-($C_1$-$C_6$)-alkoxy, ($C_1$-$C_8$)-hydroxyalkyl, ($C_6$-$C_{16}$)-aryloxy-($C_1$-$C_8$)-alkyl, ($C_7$-$C_{16}$)-aralkoxy-($C_1$-$C_8$)-alkyl, ($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_8$)-alkoxy-($C_1$-$C_6$)-alkyl, ($C_7$-$C_{12}$)-aralkyloxy-($C_1$-$C_8$)-alkoxy-($C_1$-$C_6$)-alkyl, -O-$[CH_2]_xC_fH_{(2f+1-g)}F_g$, -$OCF_2Cl$, -$OCF_2$-CHFCl, ($C_1$-$C_{12}$)-alkylcarbonyl, ($C_3$-$C_8$)-cycloalkylcarbonyl, ($C_6$-$C_{12}$)-arylcarbonyl, ($C_7$-$C_{16}$)-aralkylcarbonyl, ($C_1$-$C_{12}$)-alkoxycarbonyl, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)-alkoxycarbonyl, ($C_6$-$C_{12}$)-aryloxycarbonyl, ($C_7$-$C_{16}$)-aralkoxycarbonyl, ($C_3$-$C_8$)-cycloalkoxycarbonyl, ($C_2$-$C_{12}$)-alkenyloxycarbonyl, ($C_2$-$C_{12}$)-alkynyloxycarbonyl, ($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_6$)-alkoxycarbonyl,

($C_7$-$C_{16}$)-aralkoxy-($C_1$-$C_6$)-alkoxycarbonyl, ($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_6$)-alkoxycarbonyl, ($C_3$-$C_8$)-cycloalkoxy-($C_1$-$C_6$)-alkoxycarbonyl, ($C_1$-$C_{12}$)-alkylcarbonyloxy, ($C_3$-$C_8$)-cycloalkylcarbonyloxy, ($C_6$-$C_{12}$)-arylcarbonyloxy, ($C_7$-$C_{16}$)-aralkylcarbonyloxy, cinnamoyloxy, ($C_2$-$C_{12}$)-alkenylcarbonyloxy, ($C_2$-$C_{12}$)-alkynylcarbonyloxy, ($C_1$-$C_{12}$)-alkoxycarbonyloxy, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)-alkoxycarbonyloxy, ($C_6$-$C_{12}$)-aryloxycarbonyloxy, ($C_7$-$C_{16}$)-aralkyloxycarbonyloxy, ($C_3$-$C_8$)-cycloalkoxycarbonyloxy, ($C_2$-$C_{12}$)-alkenyloxycarbonyloxy, ($C_2$-$C_{12}$)-alkynyloxycarbonyloxy, carbamoyl, N-($C_1$-$C_{12}$)-alkylcarbamoyl, N,N-di($C_1$-$C_{12}$)-alkylcarbamoyl, N-($C_3$-$C_8$)-cycloalkylcarbamoyl, N,N-dicyclo-($C_3$-$C_8$)-alkylcarbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_3$-$C_8$)-cycloalkylcarbamoyl, N-(($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_6$)-alkyl)carbamoyl, N-($C_1$-$C_6$)-alkyl-N-(($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_6$)-alkyl)carbamoyl, N-(+)-dehydroabietylcarbamoyl, N-($C_1$-$C_6$)-alkyl-N-(+)-dehydroabietylcarbamoyl, N-($C_6$-$C_{12}$)-arylcarbamoyl, N-($C_7$-$C_{16}$)-aralkylcarbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_6$-$C_{16}$)-arylcarbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_7$-$C_{16}$)-aralkylcarbamoyl, N-(($C_1$-$C_{16}$)-alkoxy-($C_1$-$C_{10}$)-alkyl)carbamoyl, N-(($C_6$-$C_{16}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)carbamoyl, N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)carbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-(($C_1$-$C_{10}$)-alkoxy-($C_1$-$C_{10}$)-alkyl)carbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-(($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)carbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, CON($CH_2$)$_h$, in which a $CH_2$ group can be replaced by, O, S, N-($C_1$-$C_8$)-alkylimino, N-($C_3$-$C_8$)-cycloalkylimino, N-($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_4$)-alkylimino, N-($C_6$-$C_{12}$)-arylimino, N-($C_7$-$C_{16}$)-aralkylimino, N-($C_1$-$C_4$)-alkoxy-($C_1$-$C_6$)-alkylimino, and h is from 3 to 7; carbamoyloxy, N-($C_1$-$C_{12}$)-alkylcarbamoyloxy, N,N-di-($C_1$-$C_{12}$)-alkylcarbamoyloxy, N-($C_3$-$C_8$)-cycloalkylcarbamoyloxy, N-($C_6$-$C_{16}$)-arylcarbamoyloxy, N-($C_7$-$C_{16}$)-aralkylcarbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-($C_6$-$C_{12}$)-arylcarbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-($C_7$-$C_{16}$)-aralkylcarbamoyloxy, N-(($C_1$-$C_{10}$)-alkyl)carbamoyloxy, N-(($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)carbamoyloxy, N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)carbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-(($C_1$-$C_{10}$)-alkoxy-($C_1$-$C_{10}$)-alkyl)carbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-(($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)carbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)carbamoyloxy, amino, ($C_1$-$C_{12}$)-alkylamino, di-($C_1$-$C_{12}$)-alkylamino, ($C_3$-$C_8$)-cycloalkylamino, ($C_3$-$C_{12}$)-alkenylamino, ($C_3$-$C_{12}$)-alkynylamino, N-($C_6$-$C_{12}$)-arylamino, N-($C_7$-$C_{11}$)-aralkylamino, N-alkyl-aralkylamino, N-alkyl-arylamino, ($C_1$-$C_{12}$)-alkoxyamino, ($C_1$-$C_{12}$)-alkoxy-N-($C_1$-$C_{10}$)-alkylamino, ($C_1$-$C_{12}$)-alkanoylamino, ($C_3$-$C_8$)-cycloalkanoylamino, ($C_6$-$C_{12}$)-aroylamino, ($C_7$-$C_{16}$)-aralkanoylamino, ($C_1$-$C_{12}$)-alkanoyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_3$-$C_8$)-cycloalkanoyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_6$-$C_{12}$)-aroyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_7$-$C_{11}$)-aralkanoyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_1$-$C_{12}$)-alkanoylamino-($C_1$-$C_8$)-alkyl, ($C_3$-$C_8$)-cycloalkanoylamino-($C_1$-$C_8$)-alkyl, ($C_6$-$C_{12}$)-aroylamino- ($C_1$-$C_8$)-alkyl, ($C_7$-$C_{16}$)-aralkanoylamino-($C_1$-$C_8$)-alkyl, amino-($C_1$-$C_{10}$)-alkyl, N-($C_1$-$C_{10}$)-alkylamino-($C_1$-$C_{10}$)-alkyl, N,N-di-($C_1$-$C_{10}$)-alkylamino-($C_1$-$C_{10}$)-alkyl, ($C_3$-$C_8$)-cycloalkylamino-($C_1$-$C_{10}$)-alkyl, ($C_1$-$C_{12}$)-alkylmercapto, ($C_1$-$C_{12}$)-alkylsulfinyl, ($C_1$-$C_{12}$)-

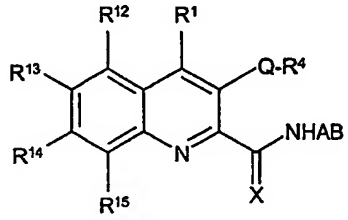
alkylsulfonyl, ($C_6$-$C_{16}$)-arylmercapto, ($C_6$-$C_{16}$)-arylsulfinyl, ($C_6$-$C_{16}$)-arylsulfonyl, ($C_7$-$C_{16}$)-aralkylmercapto, ($C_7$-$C_{16}$)-aralkylsulfinyl, or ($C_7$-$C_{16}$)-aralkylsulfonyl;

or wherein $R^1$ and $R^2$, or $R^2$ and $R^3$ form a chain $[CH_2]_o$, which is saturated or unsaturated by a C=C double bond, in which 1 or 2 $CH_2$ groups are optionally replaced by O, S, SO, $SO_2$, or NR', and R' is hydrogen, ($C_6$-$C_{12}$)-aryl, ($C_1$-$C_8$)-alkyl, ($C_1$-$C_8$)-alkoxy-($C_1$-$C_8$)-alkyl, ($C_7$-$C_{12}$)-aralkoxy-($C_1$-$C_8$)-alkyl, ($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_8$)-alkyl, ($C_1$-$C_{10}$)-alkanoyl, optionally substituted ($C_7$-$C_{16}$)-aralkanoyl, or optionally substituted (C6-C12)-aroyl; and o is 3, 4 or 5;

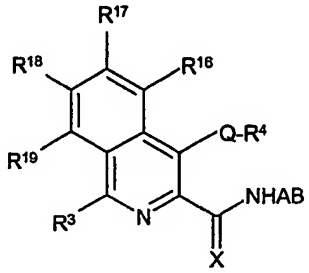
or wherein the radicals $R^1$ and $R^2$, or $R^2$ and $R^3$, together with the pyridine or pyridazine carrying them, form a 5,6,7,8-tetrahydroisoquinoline ring, a 5,6,7,8-tetrahydroquinoline ring, or a 5,6,7,8-tetrahydrocinnoline ring;

or wherein $R^1$ and $R^2$, or $R^2$ and $R^3$ form a carbocyclic or heterocyclic 5- or 6-membered aromatic ring;

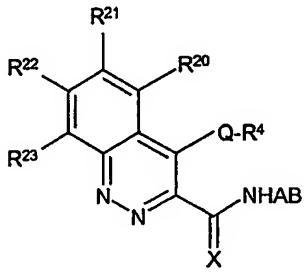
or where $R^1$ and $R^2$, or $R^2$ and $R^3$, together with the pyridine or pyridazine carrying them, form an optionally substituted heterocyclic ring systems selected from thienopyridines, furanopyridines, pyridopyridines, pyrimidinopyridines, imidazopyridines, thiazolopyridines, oxazolopyridines, quinoline, isoquinoline, and cinnoline; where quinoline, isoquinoline or cinnoline preferably satisfy the formulae Ia, Ib and Ic:




(Ia)



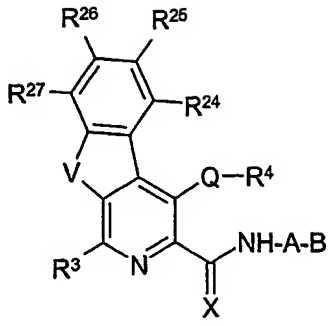

(Ib)




(Ic)

and the substituents $R^{12}$ to $R^{23}$ in each case independently of each other have the meaning of $R^1$, $R^2$ and $R^3$;

or wherein the radicals $R^1$ and $R^2$, together with the pyridine carrying them, form a compound of Formula 1d:




(Id)

where V is S, O, or $NR^k$, and $R^k$ is selected from hydrogen, $(C_1-C_6)$-alkyl, aryl, or benzyl; where an aryl radical may be optionally substituted by 1 to 5 substituents as defined above; and

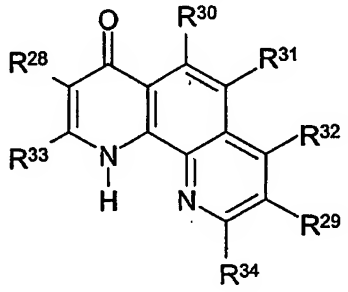
$R^{24}$, $R^{25}$, $R^{26}$, and $R^{27}$ in each case independently of each other have the meaning of $R^1$, $R^2$ and $R^3$;

f is 1 to 8;
g is 0 or 1 to (2f+1);
x is 0 to 3; and
h is 3 to 7;
including the physiologically active salts and prodrugs derived therefrom.

**[0034]** In some embodiments, compounds of Formula (I) as defined above include, but are not limited to, [(3-methoxy-pyridine-2-carbonyl)-amino]-acetic acid; 3-methoxypyridine-2-carboxylic acid N-(((hexadecyloxy)-carbonyl)-methyl)-amide hydrochloride; 3-methoxypyridine-2-carboxylic acid N-(((1-octyloxy)-carbonyl)-methyl)-amide; 3-methoxypyridine-2-carboxylic acid N-(((hexyloxy)-carbonyl)-methyl)-amide; 3-methoxypyridine-2-carboxylic acid N-(((butyloxy)-carbonyl)-methyl)-amide; 3-methoxypyridine-2-carboxylic acid N-(((2-nonyloxy)-carbonyl)-methyl)-amide racemate; 3-methoxypyridine-2-carboxylic acid N-(((heptyloxy)-carbonyl)-methyl)-amide; 3-benzyloxypyridine-2-carboxylic acid N-(((octyloxy)-carbonyl)-methyl)-amide; 3-benzyloxypyridine-2-carboxylic acid N-(((butyloxy)-carbonyl)-methyl)-amide; 5-(((3-(1-butyloxy)-propyl)-amino)-carbonyl)-3-methoxypyridine-2-carboxylic acid N-((benzyloxycarbonyl)-methyl)-amide; 5-(((3-(1-butyloxy)-propyl)-amino)-carbonyl)-3-methoxypyridine-2-carboxylic acid N-(((1-butyloxy)-carbonyl)-methyl)-amide; 5-(((3-lauryloxy)-propyl)amino)-carbonyl)-3-methoxypyridine-2-carboxylic acid N-(((benzyloxy)-carbonyl)-methyl)-amide, [(3-hydroxy-pyridine-2-carbonyl)-amino]-acetic acid; and [(3-methoxy-pyridine-2-carbonyl)-amino]-acetic acid. In other embodiments, compounds of

Formula (Ia) as defined above include, but are not limited to, N-((3-Hydroxy-6-isopropoxy-quinoline-2-carbonyl)-amino)-acetic acid, N-((6-(1-butyloxy)-3-hydroxyquinolin-2-yl)-carbonyl)-glycine, [(3-hydroxy-6-trifluoromethoxy-quinoline-2-carbonyl)-amino]-acetic acid, N-((6-chloro-3-hydroxyquinolin-2-yl)-carbonyl)-glycine, N-((7-chloro-3-hydroxyquinolin-2-yl)-carbonyl)-glycine, and [(6-chloro-3-hydroxy-quinoline-2-carbonyl)-amino]-acetic acid. In still other embodiments, the compounds of Formula (Ib) as defined above include, but are not limited to, N-((1-chloro-4-hydroxy-7-(2-propyloxy) isoquinolin-3-yl)-carbonyl)-glycine, N-((1-chloro-4-hydroxy-6-(2-propyloxy) isoquinolin-3-yl)-carbonyl)-glycine, N-((1-chloro-4-hydroxy-isoquinoline-3-carbonyl)-amino)-acetic acid, N-((1-chloro-4-hydroxy-7-methoxyisoquinolin-3-yl)-carbonyl)-glycine, N-((1-chloro-4-hydroxy-6-methoxyisoquinolin-3-yl)-carbonyl)-glycine, N-((7-butyloxy)-1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine, N-((6-benzyloxy-1-chloro-4-hydroxy-isoquinoline-3-carbonyl)-amino)-acetic acid, ((7-benzyloxy-1-chloro-4-hydroxy-isoquinoline-3-carbonyl)-amino)-acetic acid methyl ester, N-((7-benzyloxy-1-chloro-4-hydroxy-isoquinoline-3-carbonyl)-amino)-acetic acid, N-((8-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine, N-((7-butoxy-4-hydroxy-isoquinoline-3-carbonyl)-amino)-acetic acid.

[0035] In other embodiments, compounds used in the methods of the invention are selected from a compound of the formula (II)




(II)

where

$R^{28}$ is hydrogen, nitro, amino, cyano, halogen, ($C_1$-$C_4$)-alkyl, carboxy or a metabolically labile ester derivative thereof; ($C_1$-$C_4$)-alkylamino, di-($C_1$-$C_4$)-alkylamino, ($C_1$-$C_6$)-alkoxycarbonyl, ($C_2$-$C_4$)-alkanoyl, hydroxy-($C_1$-$C_4$)-alkyl, carbamoyl, N-($C_1$-$C_4$)-alkylcarbamoyl, ($C_1$-$C_4$)-alkylthio, ($C_1$-$C_4$)-alkylsulfinyl, ($C_1$-$C_4$)-alkylsulfonyl, phenylthio, phenylsulfinyl, phenylsulfonyl, said phenyl or phenyl groups being optionally substituted with 1 to 4 identical or different halogen, ($C_1$-$C_4$)-alkyoxy, ($C_1$-$C_4$)-alkyl, cyano, hydroxy, trifluoromethyl, fluoro-($C_1$-$C_4$)-alkylthio, fluoro-($C_1$-$C_4$)-alkylsulfinyl, fluoro-($C_1$-$C_4$)-alkylsulfonyl, ($C_1$-$C_4$)-alkoxy-($C_2$-$C_4$)-alkoxycarbonyl, N,N-di-[($C_1$-$C_4$)-alkyl]carbamoyl-($C_1$-$C_4$)-alkoxycarbonyl, ($C_1$-$C_4$)-alkylamino-($C_2$-$C_4$)-alkoxycarbonyl, di-($C_1$-$C_4$)-alkylamino-($C_2$-$C_4$)-alkoxycarbonyl, ($C_1$-$C_4$)-

alkoxy-($C_2$-$C_4$)-alkoxy-($C_2$-$C_4$)-alkoxycarbonyl, ($C_2$-$C_4$)-alkanoyloxy-$C_1$-$C_4$)-alkyl, or N-[amino-($C_2$-$C_8$)-alkyl]-carbamoyl;

$R^{29}$ is hydrogen, hydroxy, amino, cyano, halogen, ($C_1$-$C_4$)-alkyl, carboxy or metabolically labile ester derivative thereof, ($C_1$-$C_4$)-alkylamino, di-($C_1$-$C_4$)-alkylamino, ($C_1$-$C_6$)-alkoxycarbonyl, ($C_2$-$C_4$)-alkanoyl, ($C_1$-$C_4$)-alkoxy, carboxy-($C_1$-$C_4$)-alkoxy, ($C_1$-$C_4$)-alkoxycarbonyl-($C_1$-$C_4$)-alkoxy, carbamoyl, N-($C_1$-$C_8$)-alkylcarbamoyl, N,N-di-($C_1$-$C_8$)-alkylcarbamoyl, N-[amino-($C_2$-$C_8$)-alkyl)-carbamoyl, N-[($C_1$-$C_4$)-alkylamino-($C_1$-$C_8$)-alkyl]-carbamoyl, N-[di-($C_1$-$C_4$)-alkylamino-($C_1$-$C_8$)-alkyl)]-carbamoyl, N-cyclohexylcarbamoyl, N-[cyclopentyl]-carbamoyl, N-($C_1$-$C_4$)-alkylcyclohexylcarbamoyl, N-($C_1$-$C_4$)-alkylcyclopentylcarbamoyl, N-phenylcarbamoyl, N-($C_1$-$C_4$)-alkyl-N-phenylcarbamoyl, N,N-diphenylcarbamoyl, N-[phenyl-($C_1$-$C_4$)-alkyl]-carbamoyl, N-($C_1$-$C_4$)-alkyl-N-[phenyl-($C_1$-$C_4$)-alkyl]-carbamoyl, or N,N-di-[phenyl-($C_1$-$C_4$)-alkyl]-carbamoyl, said phenyl or phenyl groups being optionally substituted with 1 to 4 identical or different halogen, ($C_1$-$C_4$)-alkyoxy, ($C_1$-$C_4$)-alkyl, cyano, hydroxy, trifluoromethyl, N-[($C_2$-$C_4$)-alkanoyl]-carbamoyl, N-[($C_1$-$C_4$)-alkoxycarbonyl]-carbamoyl, N-[fluoro-($C_2$-$C_6$)-alkyl]-carbamoyl, N,N-[fluoro-($C_2$-$C_6$)-alkyl]-N-($C_1$-$C_4$)-alkylcarbamoyl, N,N-[di-fluoro-($C_2$-$C_6$)-alkyl]carbamoyl, pyrrolidin-1-ylcarbonyl, piperidinocarbonyl, piperazin-1-ylcarbonyl, morpholinocarbonyl, wherein the heterocyclic group, is optionally substituted with 1 to 4, ($C_1$-$C_4$)-alkyl, benzyl , 1,2,3,4-tetrahydro-isoquinolin-2-ylcarbonyl, N,N-[di-($C_1$-$C_4$)-alkyl]-thiocarbamoyl, N-($C_2$-$C_4$)-alkanoylamino, or N-[($C_1$-$C_4$)-alkoxycarbonyl]-amino;

$R^{30}$ is hydrogen, ($C_1$-$C_4$)-alkyl, ($C_2$-$C_4$)-alkoxy, halo, nitro, hydroxy, fluoro-(1-4C)alkyl, or pyridinyl;

$R^{31}$ is hydrogen, ($C_1$-$C_4$)-alkyl, ($C_2$-$C_4$)-alkoxy, halo, nitro, hydroxy, fluoro-($C_1$-$C_4$)-alkyl, pyridinyl, or methoxy;

$R^{32}$ is hydrogen, hydroxy, amino, ($C_1$-$C_4$)-alkylamino, di-($C_1$-$C_4$)-alkylamino, halo, ($C_1$-$C_4$)-alkoxy-($C_2$-$C_4$)-alkoxy, fluoro-($C_1$-$C_6$)-alkoxy, pyrrolidin-1-yl, piperidino, piperazin-1-yl, or morpholino, wherein the heterocyclic group is optionally substituted with 1 to 4 identical or different ($C_1$-$C_4$)-alkyl or benzyl; and

$R^{33}$ and $R^{34}$ are individually selected from hydrogen, ($C_1$-$C_4$)-alkyl, and ($C_1$-$C_4$)-alkoxy; including pharmaceutically-acceptable salts and pro-drugs derived therefrom.

[0036] In some embodiments, compounds of Formula (II) as defined above include, but are not limited to, 4-oxo-1,4-dihydro-[1,10]phenanthroline-3-carboxylic acid, 3-carboxy-5-hydroxy-4-oxo-3,4-dihydro-1,10-phenanthroline, 3-carboxy-5-methoxy-4-oxo-3,4-dihydro-1,10-phenanthroline, 5-methoxy-4-oxo-1,4-dihydro-[1,10]phenanthroline-3-carboxylic acid ethyl ester, 5-methoxy-4-oxo-1,4-dihydro-[1,10]phenanthroline-3-carboxylic acid, and 3-carboxy-8-hydroxy-4-oxo-3,4-dihydro-1,10-phenanthroline.

[0037] The compounds can be administered singly or in combination with various other therapeutic approaches. In one embodiment, the compound is administered with another 2-oxoglutarate dioxygenase inhibitor, wherein the two compounds have differential specificity for individual 2-oxoglutarate dioxygenase family members. The two compounds may be administered at the same time as a ratio of one relative to the other or may be administered consecutively during a treatment time course, e.g., following myocardial infarction. In one specific embodiment, one compound specifically inhibits HIF prolyl hydroxylase activity, and a second compound specifically inhibits procollagen prolyl 4-hydroxylase activity. In another embodiment, the compound is administered with another therapeutic agent having a different mode of action, e.g., an ACE inhibitor (ACEI), angiotensin-II receptor blocker (ARB), diuretic, and/or digoxin. In yet another embodiment, the compound is administered with carnitine.

[0038] In one aspect, a compound of the invention inhibits one or more 2-oxoglutarate dioxygenase enzymes. In one embodiment, the compound inhibits at least two 2-oxoglutarate dioxygenase family members, e.g., HIF prolyl hydroxylase and procollagen prolyl 4-hydroxylase, with either the same specificity or with differential specificity. In another embodiment, the compound is specific for one 2-oxoglutarate dioxygenase, e.g., HIF prolyl hydroxylase, and shows little to no specificity for other family members.

[0039] Preferred embodiments of the invention comprise methods using oral and transdermal delivery mechanisms. Thus, the present invention also provides an oral formulation comprising a compound of the invention. In another preferred embodiment, the present methods involve transdermal administration of a compound of the invention. Thus, the present invention also provides a transdermal patch or pad comprising a compound of the invention.

[0040] These and other embodiments of the subject invention will readily occur to those of skill in the art in light of the disclosure herein, and all such embodiments are specifically contemplated.

## BRIEF DESCRIPTION OF THE DRAWINGS

[0041] Figures 1A and 1B show HIF-1α stabilization in cells treated with compounds of the invention. Figure 1A shows stabilization and accumulation of HIF-1α in human foreskin fibroblasts (HFF) treated with various compounds of the invention. Figure 1B shows a dose response for HIF-1α stabilization and accumulation in different human cells treated with a compound of the invention. Cell lines shown in the figure include HFF, human microvascular endothelial cells (HMEC), venous endothelium (AG7), human umbilical vein endotheial cells (HUVEC), squamous cell carcinoma (SCC), human lung fibroblasts (HLF), mammary gland epithelial adenocarcinoma (MCF7), transformed fetal kidney cells (293A), and cervical adenocarcinoma cells (HeLa).

[0042] Figures 2A and 2B show HIF-1α stabilization and accumulation in human cells treated with compounds of the invention. Figure 2A shows 293A and human hepatocarcinoma cells (Hep3B) treated with various compounds of the invention. Figure 2B shows a dose response for HIF-1α stabilization in Hep3B cells treated with exemplary compounds of the invention.

[0043] Figures 3A and 3B show oxygen consumption and cell viability in human cells treated with compounds of the invention. Figure 3A shows single-dose and dose-response oxygen consumption in cells treated with various compounds of the invention. Figure 3B shows cell proliferation and viability as measured by cleavage of WST-1 tetrazolium salt (Roche Diagnostics Corp., Indianapolis IN) in cells treated with selected compounds from Figure 3A.

[0044] Figures 4A and 4B show increased expression of HIF-responsive genes in human cells treated with compounds of the invention. Figure 4A shows levels of vascular endothelial growth factor (VEGF), a key gene in blood vessel formation, in human cell culture media following treatment with compounds of the invention. Cell lines shown in the figure are 293A, Hep3B, and HFF. Figure 4B shows a time course for increase in aldolase, a key enzyme in the glycolytic pathway, in cells treated with a compound of the invention.

[0045] Figures 5A and 5B show increase in expression of angiogenic proteins in the lung of animals treated with a compound of the invention. Figure 5A shows a montage of angiogenic gene expression. Genes represented in the figure include vascular endothelial growth factor (VEGF)-C, Flt-1/VEGF receptor-1, adrenomedullin, endothelin-1, plasminogen

activator inhibitor (PAI)-1, and Cyr61. Figure 5B shows expression of genes encoding endothelin-1 and adrenomedullin selected from Figure 5A.

[0046] Figures 6A and 6B show increased expression of HIF-responsive genes *in vivo*. Figure 6A shows increased levels of transcript encoding VEGF in liver and kidney of mice treated with compounds of the invention. Figure 6B shows levels of VEGF in mouse plasma at 2, 5, and 20 hours following final treatment with a compound of the invention relative to an untreated control group.

[0047] Figures 7A and 7B show increase in expression of glycolytic enzymes in the kidney of animals treated with a compound of the invention. Figure 7A shows a montage of glycolytic gene expression. Genes represented in the figure include aldolase-A, enolase-1, Glut1, Glut3, GAPDH, hexokinase-1 and -2, lactate dehydrogenase-A, phosphofructokinase-L and -C, phosphoglycerate kinase-1, and pyruvate kinase-M. Figure 7B shows expression of genes encoding aldolase-A and phosphofructokinase-L selected from Figure 7A.

[0048] Figure 8 shows percent survival in a group treated with a compound of the invention (n=34) compared to an untreated group (n=34) at time intervals following induced myocardial infarction.

[0049] Figures 9A and 9B show improvement in cardiac architecture following myocardial infarction in animals treated with a compound of the invention relative to untreated controls. Figure 9A shows changes in the left ventricular end systolic diameter (LVESD) in a group treated with a compound of the invention relative to an untreated group at time intervals following induced myocardial infarction. Figure 9B shows changes in the left ventricular end diastolic diameter (LVEDD) in a group treated with a compound of the invention relative to an untreated group at time intervals following induced myocardial infarction.

[0050] Figures 10A and 10B show improvement in cardiac performance following myocardial infarction in animals treated with a compound of the invention relative to untreated controls. Figure 10A shows changes in the left ventricular ejection fraction in a group treated with a compound of the invention relative to an untreated group at time intervals following induced myocardial infarction. Figure 10B shows changes in the fractional shortening in a group treated with a compound of the invention relative to an untreated group at time intervals following induced myocardial infarction.

[0051] Figure 11 shows the contractile response of the heart 4 weeks post-MI in a group treated with a compound of the invention relative to an untreated group with and without an isoproterenol challenge.

[0052] Figures 12A and 12B show improvements to heart architecture following myocardial infarction in animals pretreated with a compound of the invention relative to untreated controls. Figure 12A shows statistically significant improvement ($p<0.05$) in fractional shortening in treated animals relative to untreated controls one week after induced myocardial infarction. Figure 12B shows statistically significant improvement in left ventricle end-diastolic diameter (LVEDD; $p<0.005$) and left ventricular end-systolic diameter (LVESD; $p<0.001$) in treated animals relative to untreated controls one week after induced myocardial infarction.

[0053] Figure 13 shows increased survivability in animals subjected to renal ischemic-reperfusion injury that have been pretreated and consequently treated with compounds of the invention relative to untreated and sham-operated controls.

[0054] Figure 14A and 14B show improvement in kidney function following ischemic-reperfusion injury in animals pretreated with a compound of the invention relative to untreated controls. Figure 14A shows lower blood urea nitrogen levels in treated animals relative to untreated controls at 3 and 7 days after inducing ischemia-reperfusion injury. Figure 14B shows lower blood cholesterol levels in treated animals relative to untreated controls at 3, 7, and 14 days after inducing ischemia-reperfusion injury.

[0055] Figures 15A and 15B show improved healing of chronic wounds in animals treated with a compound of the invention relative to untreated controls. Figure 15A shows increased epithelialization and formation of granulation tissue in treated animals relative to untreated controls 7 and 10 days after induction of wounds. Figure 15B shows no difference in peak-peak distance within the scar in treated animals relative to untreated controls.

## DESCRIPTION OF THE INVENTION

[0056] Before the present compositions and methods are described, it is to be understood that the invention is not limited to the particular methodologies, protocols, cell lines, assays, and reagents described, as these may vary. It is also to be understood that the terminology used herein is intended to describe particular embodiments of the present

invention, and is in no way intended to limit the scope of the present invention as set forth in the appended claims.

[0057] It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural references unless context clearly dictates otherwise. Thus, for example, a reference to "a fragment" includes a plurality of such fragments; a reference to an "antibody" is a reference to one or more antibodies and to equivalents thereof known to those skilled in the art, and so forth.

[0058] Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and materials are now described. All publications cited herein are incorporated herein by reference in their entirety for the purpose of describing and disclosing the methodologies, reagents, and tools reported in the publications that might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

[0059] The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, molecular biology, cell biology, genetics, immunology and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Gennaro, A.R., ed. (1990) Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co.; Hardman, J.G., Limbird, L.E., and Gilman, A.G., eds. (2001) The Pharmacological Basis of Therapeutics, 10th ed., McGraw-Hill Co.; Colowick, S. et al., eds., Methods In Enzymology, Academic Press, Inc.; Weir, D.M., and Blackwell, C.C., eds. (1986) Handbook of Experimental Immunology, Vols. I-IV, Blackwell Scientific Publications; Maniatis, T. et al., eds. (1989) Molecular Cloning: A Laboratory Manual, 2nd edition, Vols. I-III, Cold Spring Harbor Laboratory Press; Ausubel, F.M. et al., eds. (1999) Short Protocols in Molecular Biology, 4th edition, John Wiley & Sons; Ream et al., eds. (1998) Molecular Biology Techniques: An Intensive Laboratory Course, Academic Press; Newton, C.R., and Graham, A., eds. (1997) PCR (Introduction to Biotechniques Series), 2nd ed., Springer Verlag.

**DEFINITIONS**

[0060] The term "ischemia" refers to a reduction in blood flow. Ischemia is associated with a reduction in nutrients, including oxygen, delivered to tissues. Ischemia may

arise due to conditions such as atherosclerosis, formation of a thrombus in an artery or vein, or blockage of an artery or vein by an embolus, vascular closure due to other causes, e.g., vascular spasm, etc. Such conditions may reduce blood flow, producing a state of hypoperfusion to an organ or tissue, or block blood flow completely. Other conditions that can produce ischemia include tissue damage due to trauma or injury, such as, e.g., spinal cord injury; viral infection, which can lead to, e.g., congestive heart failure, etc. The terms "ischemic conditions" and "ischemic disorders" refer to acute ischemic conditions including, but not limited to, myocardial infarction, ischemic stroke, pulmonary embolism, perinatal hypoxia, circulatory shock including, e.g., hemorrhagic, septic, cardiogenic, etc., mountain sickness, acute respiratory failure, etc., chronic ischemic conditions including atherosclerosis, chronic venous insufficiency, chronic heart failure, cardiac cirrhosis, diabetes, macular degeneration, sleep apnea, Raynaud's disease, systemic sclerosis, nonbacterial thrombotic endocarditis, occlusive artery disease, angina pectoris, TIAs, chronic alcoholic liver disease, etc. Ischemic conditions may also result when individuals are placed under general anesthesia, and can cause tissue damage in organs prepared for transplant.

[0061] The terms "hypoxia" and "hypoxic" refer to an environment with levels of oxygen below normal. Hypoxia may be induced in cells by culturing the cells in a reduced oxygen environment, or cells may be treated with compounds that mimic hypoxia. Determining oxygen levels that define hypoxia in cell culture is well within the skill in the art.

[0062] The terms "hypoxic conditions" and "hypoxic disorders" include, but are not limited to, ischemic disorders (ischemic hypoxia) such as those listed above, wherein hypoxia results from reduced circulation; pulmonary disorders (hypoxic hypoxia) such as COPD, severe pneumonia, pulmonary edema, pulmonary hypertension, hyaline membrane disease, and the like, wherein hypoxia results from reduced oxygenation of the blood in the lungs; anemic disorders (anemic hypoxia) such as gastric or duodenal ulcers, liver or renal disease, thrombocytopenia or blood coagulation disorders, cancer or other chronic illness, cancer chemotherapy and other therapeutic interventions that produce anemia, and the like, wherein hypoxia results from a decreased concentration of hemoglobin or red blood cells; and altitude sickness, etc.

[0063] The terms "disorders" and "diseases" are used inclusively and refer to any condition deviating from normal. The terms "ischemic conditions" and "ischemic disorders" refer to any condition, disease, or disorder that is associated with ischemia. The terms "hypoxic conditions" and "hypoxic disorders" refer to any condition, disease, or disorder that

is associated with hypoxia. Such ischemic and hypoxic disorders include, but are not limited to, those disorders described above.

[0064] The term "HIFα" refers to the alpha subunit of hypoxia inducible factor protein. HIFα may be any human or other mammalian protein, or fragment thereof, including, but not limited to, human HIF-1α (Genbank Accession No. Q16665), HIF-2α (Genbank Accession No. AAB41495), and HIF-3α (Genbank Accession No. AAD22668); murine HIF-1α (Genbank Accession No. Q61221), HIF-2α (Genbank Accession No. BAA20130 and AAB41496), and HIF-3α (Genbank Accession No. AAC72734); rat HIF-1α (Genbank Accession No. CAA70701), HIF-2α (Genbank Accession No. CAB96612), and HIF-3α (Genbank Accession No. CAB96611); and cow HIF-1α (Genbank Accession No. BAA78675). HIFα may also be any non-mammalian protein or fragment thereof, including Xenopus laevis HIF-1α (Genbank Accession No. CAB96628), Drosophila melanogaster HIF-1α (Genbank Accession No. JC4851), and chicken HIF-1α (Genbank Accession No. BAA34234). HIFα gene sequences may also be obtained by routine cloning techniques, for example, by using all or part of a HIFα gene sequence described above as a probe to recover and determine the sequence of a HIFα gene in another species.

[0065] Fragments of HIFα include the regions defined by human HIF-1α from amino acid 401 to 603 (Huang et al., *supra*), amino acid 531 to 575 (Jiang et al. (1997) J Biol Chem 272:19253-19260), amino acid 556 to 575 (Tanimoto et al., *supra*), amino acid 557 to 571 (Srinivas et al. (1999) Biochem Biophys Res Commun 260:557-561), and amino acid 556 to 575 (Ivan and Kaelin (2001) Science 292:464-468). Further, a fragment of HIFα includes any fragment containing at least one occurrence of the motif LXXLAP, e.g., as occurs in the HIF-1α native sequence at $L_{397}$TLLAP and $L_{559}$EMLAP. Additionally, a fragment of HIFα includes any fragment retaining at least one functional or structural characteristic of HIFα. For example, a HIF peptide for use in the screening assay of Example 7 may comprise [methoxycoumarin]-DLDLEALAPYIPADDDFQL-amide (SEQ ID NO:5).

[0066] The terms "HIF prolyl hydroxylase" and "HIF PH" refer to any enzyme capable of hydroxylating a proline residue in the HIF protein. Preferably, the proline residue hydroxylated by HIF PH includes the proline found within the motif LXXLAP, e.g., as occurs in the human HIF-1α native sequence at $L_{397}$TLLAP and $L_{559}$EMLAP. HIF PH includes members of the Egl-Nine (EGLN) gene family described by Taylor (2001,Gene 275:125-132), and characterized by Aravind and Koonin (2001, Genome Biol 2:RESEARCH0007), Epstein et al. (2001, Cell 107:43-54), and Bruick and McKnight (2001, Science 294:1337-1340). Examples of HIF PH enzymes include human SM-20 (EGLN1) (GenBank

Accession No. AAG33965; Dupuy et al. (2000) Genomics 69:348-54), EGLN2 isoform 1 (GenBank Accession No. CAC42510; Taylor, *supra*), EGLN2 isoform 2 (GenBank Accession No. NP_060025), and EGLN3 (GenBank Accession No. CAC42511; Taylor, *supra*); mouse EGLN1 (GenBank Accession No. CAC42515), EGLN2 (GenBank Accession No. CAC42511), and EGLN3 (SM-20) (GenBank Accession No. CAC42517); and rat SM-20 (GenBank Accession No. AAA19321). Additionally, HIF PH may include *Caenorhabditis elegans* EGL-9 (GenBank Accession No. AAD56365) and *Drosophila melanogaster* CG1114 gene product (GenBank Accession No. AAF52050). HIF PH also includes any fragment retaining at least one stuctural or function feature of the foregoing full-length proteins, including a fragment having hydroxylase activity.

[0067] The terms "amino acid sequence" or "polypeptide" as used herein, e.g., to refer to HIF$\alpha$ and fragments thereof, or HIF PH and fragments thereof, contemplate an oligopeptide, peptide, or protein sequence, or to a fragment of any of these, and to naturally occurring or synthetic molecules. "Fragments" can refer to any portion of a sequence that retains at least one structural or functional characteristic of the protein. Immunogenic fragments or antigenic fragments are fragments of polypeptides, preferably, fragments of about five to fifteen amino acids in length, that retain at least one biological or immunological activity. Where "amino acid sequence" is used to refer to the polypeptide sequence of a naturally occurring protein molecule, "amino acid sequence" and like terms are not meant to limit the amino acid sequence to the complete native sequence associated with the recited protein molecule.

[0068] The term "related proteins" as used herein, for example, to refer to proteins related to HIF$\alpha$ prolyl hydroxylase, encompasses other 2-oxoglutarate dioxygenase enzymes, especially those family members that similarly require $Fe^{2+}$, 2-oxoglutarate, and oxygen to maintain hydroxylase activity. Such enzymes include, but are not limited to, e.g., procollagen lysyl hydroxylase, procollagen prolyl 4-hydroxylase, and Factor Inhibiting HIF (FIH), an asparaginyl hydroxylase responsible for regulating transactivation of HIF$\alpha$. (GenBank Accession No. AAL27308; Mahon et al. (2001) Genes Dev 15:2675-2686; Lando et al. (2002) Science 295:858-861; and Lando et al. (2002) Genes Dev 16:1466-1471. See, also, Elkins et al. (2002) J Biol Chem C200644200.)

[0069] The term "agonist" refers to a molecule that increases or prolongs the duration of the effect of a particular molecule, e.g., an enzyme or protein, or a particular environment, e.g., hypoxia. Agonists may include proteins, nucleic acids, carbohydrates, or any other molecules that modulate the effects of the target molecule.

[0070] The term "antagonist" refers to a molecule which decreases the extent or duration of the effect of the biological or immunological activity of a particular molecule. Antagonists may include proteins, nucleic acids, carbohydrates, antibodies, or any other molecules that decrease the effect of the target molecule.

[0071] The term "microarray" refers to any arrangement of nucleic acids, amino acids, antibodies, etc., on a substrate. The substrate can be any suitable support, e.g., beads, glass, paper, nitrocellulose, nylon, or any appropriate membrane, etc. A substrate can be any rigid or semi-rigid support including, but not limited to, membranes, filters, wafers, chips, slides, fibers, beads, including magnetic or nonmagnetic beads, gels, tubing, plates, polymers, microparticles, capillaries, etc. The substrate can provide a surface for coating and/or can have a variety of surface forms, such as wells, pins, trenches, channels, and pores, to which the nucleic acids, amino acids, etc., may be bound.

[0072] The term "excipient" as used herein means an inert or inactive substance used in the production of pharmaceutical products or other tablets, including without limitation any substance used as a binder, disintegrant, coating, compression/encapsulation aid, cream or lotion, lubricant, parenteral, sweetener or flavoring, suspending/gelling agent, or wet granulation agent. Binders include, e.g., carbopol, povidone, xanthan gum, etc.; coatings include, e.g., cellulose acetate phthalate, ethylcellulose, gellan gum, maltodextrin, etc.; compression/encapsulation aids include, e.g., calcium carbonate, dextrose, fructose dc, honey dc, lactose (anhydrate or monohydrate; optionally in combination with aspartame, cellulose, or microcrystalline cellulose), starch dc, sucrose, etc.; disintegrants include, e.g., croscarmellose sodium, gellan gum, sodium starch glycolate, etc.; creams and lotions include, e.g., maltodextrin, carrageenans, etc.; lubricants include, e.g., magnesium stearate, stearic acid, sodium stearyl fumarate, etc.; materials for chewable tablets include, e.g., dextrose, fructose dc, lactose (monohydrate, optionally in combination with aspartame or cellulose), etc.; parenterals include, e.g., mannitol, povidone, etc.; plasticizers include, e.g., dibutyl sebacate, polyvinylacetate phthalate, etc.; suspending/gelling agents include, e.g., carrageenan, sodium starch glycolate, xanthan gum, etc.; sweeteners include, e.g., aspartame, dextrose, fructose dc, sorbitol, sucrose dc, etc.; and wet granulation agents include, e.g., calcium carbonate, maltodextrin, microcrystalline cellulose, etc.

[0073] The term "sample" is used herein in its broadest sense. Samples may be derived from any source, for example, from bodily fluids, secretions, tissues, cells, or cells in culture including, but not limited to, saliva, blood, urine, serum, plasma, vitreous, synovial

fluid, cerebral spinal fluid, amniotic fluid, and organ tissue (e.g., biopsied tissue); from chromosomes, organelles, or other membranes isolated from a cell; from genomic DNA, cDNA, RNA, mRNA, etc.; and from cleared cells or tissues, or blots or imprints from such cells or tissues. Samples may be derived from any source, such as, for example, a human subject, or a non-human mammalian subject, etc. Also contemplated are samples derived from any animal model of disease. A sample can be in solution or can be, for example, fixed or bound to a substrate. A sample can refer to any material suitable for testing for the presence of HIFα or of fragments of HIFα or suitable for screening for molecules that bind to HIFα or to fragments thereof. Methods for obtaining such samples are within the level of skill in the art.

[0074] The term "subject" is used herein in its broadest sense. Subjects may include isolated cells, either prokaryotic or eukaryotic, or tissues grown in culture. Preferably, subjects include animals, particularly a mammalian species including rat, rabbit, bovine, ovine, porcine, murine, equine, and primate, particularly human.

**INVENTION**

[0075] The present invention provides methods of stabilizing HIFα, to compounds that can be used in the methods, and to the use of the methods to prevent or treat disorders associated with HIF including, but not limited to, hypoxic and/or ischemic disorders such as those described above. The present invention further relates to the discovery that stabilization of the alpha subunit of hypoxia inducible factor (HIFα) is an effective therapeutic approach with unexpected benefits when applied to treatment or prevention of conditions associated with hypoxia and/or ischemia, e.g., myocardial infarction, stroke, occlusive arterial disease, angina pectoris, cardiac cirrhosis, atherosclerosis, etc.

[0076] The present invention contemplates methods of stabilizing HIF to augment angiogenesis, the response to acute hypoxia, and adaptation to chronic hypoxia. As tissue ischemia is a major cause of morbidity and mortality, the identification of methods that stabilize HIFα is beneficial in the treatment of hypoxic conditions. Further, the methods can be used to produce the beneficial effects of, e.g., a preconditioning hypoxic response, by stabilizing HIFα in a normoxic environment prior to an ischemic or hypoxic event. The methods can also be used to induce HIFα-specific effects, as described below, including therapeutic angiogenesis to restore blood flow to damaged tissues; neuroprotection to prevent, e.g., apoptotic loss of neurons associated with neurodegenerative diseases; and protection against oxidative damage produced by reactive oxygen species resulting from, e.g., reperfusion following an ischemic or hypoxic event.

[0077] When the methods of the invention are used to treat a disorder associated with ischemia and/or hypoxia, the disorder may be an acute ischemic disorder such as pulmonary, intestinal, cerebral, and/or myocardial infarction, or a chronic ischemic condition such as occlusive arterial disease, liver cirrhosis, congestive heart failure, etc. Further, the methods of the invention can be used to treat ischemia due to a transient or acute trauma, insult, or injury such as, e.g., a spinal cord injury, or to treat a patient diagnosed with, e.g., a pulmonary disorder such as pulmonary embolism and the like.

[0078] When the methods of the invention are used to prevent tissue damage caused by HIF-associated disorders including, but not limited to, ischemic and hypoxic disorders, treatment may be predicated on predisposing conditions, e.g., hypertension, diabetes, occlusive arterial disease, chronic venous insufficiency, Raynaud's disease, systemic sclerosis, cirrhosis, congestive heart failure, etc. Similarly, the methods of the invention can be used as a pretreatment to decrease or prevent the tissue damage caused by HIF-associated disorders including, but not limited to, ischemic and hypoxic disorders. The need for pretreatment may be based on a patient's history of recurring episodes of an ischemic condition, e.g., myocardial infarction or transient ischemic attacks; based on symptoms of impending ischemia, e.g., angina pectoris; or based on physical parameters implicating possible or likely ischemia or hypoxia, such as is the case with, e.g., individuals placed under general anesthesia or temporarily working at high altitudes. The methods may also be used in the context of organ transplants to pretreat organ donors and to maintain organs removed from the body prior to implantation in a recipient.

[0079] Presented herein is the discovery that stabilization of HIF$\alpha$ is modulated by proline hydroxylation and that HIF$\alpha$ stabilization is effective for treating or preventing the development or persistence of ischemic conditions such as DVT, angina pectoris, pulmonary embolism, stroke, myocardial infarction, etc. Specifically, it has been shown that HIF-1$\alpha$ and a HIF-1$\alpha$ peptide corresponding to residues 556 to 575 [HIF(556-575)] preincubated with rabbit reticulocyte lysate (RRL) bind specifically to the von Hippel Lindau protein (pVHL), and that such binding leads to the ubiquitination and degradation of HIF-1$\alpha$. It has also been shown that mutation of the highly conserved colinear sequence $M_{561}$LAPYIPM within HIF(556-575) to eight consecutive alanines stabilized HIF(556-575) under normoxic conditions. (Srinivas et al., *supra*.) An alanine scan of the region showed that mutation of $P_{564}$ to alanine in the context of full-length HIF-1$\alpha$ or a glutathione *S*-transferase (GST)-HIF$\alpha$ oxygen degradation domain (ODD) fusion protein (Gal4-ODD) abrogated pVHL-binding activity. The modification of $P_{564}$ was identified as an hydroxylation by electrospray ion trap tandem mass spectrometry (MS/MS),

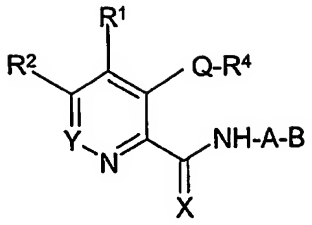
and by thin layer chromatography of Gal4-HIF(555-575) that was *in vitro* translated using RRL in the presence of [$^3$H]proline. The functional significance of the proline hydroxylation was demonstrated by showing that $P_{564}$-hydroxylated HIF$\alpha$ bound pVHL, while HIF-1$\alpha$ mutant containing a single point mutation of $P_{564}$ to alanine was stable in COS7 cells and was insensitive to the hypoxia mimetic desferrioxamine. (See Ivan and Kaelin, *supra*; Jaakkola et al. (2001) Science 292:468-472.)

**[0080]** As HIF$\alpha$ is modified by proline hydroxylation, a reaction requiring oxygen and $Fe^{2+}$, the present invention contemplates in one aspect that the enzyme responsible for HIF$\alpha$ hydroxylation is a member of the 2-oxoglutarate dioxygenase family. Such enzymes include, but are not limited to, procollagen lysyl hydroxylase, procollagen prolyl 3-hydroxylase, procollagen prolyl 4-hydroxylase $\alpha$(I) and $\alpha$(II), thymine 7-hydroxylase, aspartyl (asparaginyl) $\beta$-hydroxylase, $\epsilon$-N-trimethyllysine hydroxylase, and $\gamma$-butyrobetaine hydroxylase, etc. These enzymes require oxygen, $Fe^{2+}$, 2-oxoglutarate, and ascorbic acid for their hydroxylase activity. (See, e.g., Majamaa et al. (1985) Biochem J 229:127-133; Myllyharju and Kivirikko (1997) EMBO J 16:1173-1180; Thornburg et al. (1993) 32:14023-14033; and Jia et al. (1994) Proc Natl Acad Sci USA 91:7227-7231.)

**[0081]** Several small molecule inhibitors of prolyl 4-hydroxylase have been identified. (See, e.g., Majamaa et al., *supra*; Kivirikko and Myllyharju (1998) Matrix Biol 16:357-368; Bickel et al. (1998) Hepatology 28:404-411; Friedman et al. (2000) Proc Natl Acad Sci USA 97:4736-4741; and Franklin et al. (2001) Biochem J 353:333-338; all incorporated by reference herein in their entirety.) The present invention contemplates the use of these compounds in the methods provided herein.

**[0082]** Compounds that can be used in the methods of the invention include, for example, structural mimetics of 2-oxoglutarate. Such compounds may inhibit the target 2-oxoglutarate dioxygenase enzyme family member competitively with respect to 2-oxoglutarate and noncompetitively with respect to iron. (Majamaa et al. (1984) Eur J Biochem 138:239-245; and Majamaa et al., *supra*.)

**[0083]** In certain embodiments, compounds used in the methods of the invention are selected from a compound of the formula (I)



(I)

wherein

A is 1,2-arylidene, 1,3-arylidene, 1,4-arylidene; or ($C_1$-$C_4$)-alkylene, optionally substituted by one or two halogen, cyano, nitro, trifluoromethyl, ($C_1$-$C_6$)-alkyl, ($C_1$-$C_6$)-hydroxyalkyl, ($C_1$-$C_6$)-alkoxy, -O-$[CH_2]_x$-$C_fH_{(2f+1-g)}Hal_g$, ($C_1$-$C_6$)-fluoroalkoxy, ($C_1$-$C_8$)-fluoroalkenyloxy, ($C_1$-$C_8$)-fluoroalkynyloxy, -$OCF_2Cl$, -O-$CF_2$-CHFCl; ($C_1$-$C_6$)-alkylmercapto, ($C_1$-$C_6$)-alkylsulfinyl, ($C_1$-$C_6$)-alkylsulfonyl, ($C_1$-$C_6$)-alkylcarbonyl, ($C_1$-$C_6$)-alkoxycarbonyl, carbamoyl, N-($C_1$-$C_4$)-alkylcarbamoyl, N,N-di-($C_1$-$C_4$)-alkylcarbamoyl, ($C_1$-$C_6$)-alkylcarbonyloxy, ($C_3$-$C_8$)-cycloalkyl, phenyl, benzyl, phenoxy, benzyloxy, anilino, N-methylanilino, phenylmercapto, phenylsulfonyl, phenylsulfinyl, sulfamoyl, N-($C_1$-$C_4$)-alkylsulfamoyl, N,N-di-($C_1$-$C_4$)-alkylsulfamoyl; or by a substituted ($C_6$-$C_{12}$)-aryloxy, ($C_7$-$C_{11}$)-aralkyloxy, ($C_6$-$C_{12}$)-aryl, ($C_7$-$C_{11}$)-aralkyl radical, which carries in the aryl moiety one to five identical or different substituents selected from halogen, cyano, nitro, trifluoromethyl, ($C_1$-$C_6$)-alkyl, ($C_1$-$C_6$)-alkoxy, -O-$[CH_2]_x$-$C_fH_{(2f+1-g)}Hal_g$, -$OCF_2Cl$, -O-$CF_2$-CHFCl, ($C_1$-$C_6$)-alkylmercapto, ($C_1$-$C_6$)-alkylsulfinyl, ($C_1$-$C_6$)-alkylsulfonyl, ($C_1$-$C_6$)-alkylcarbonyl, ($C_1$-$C_6$)-alkoxycarbonyl, carbamoyl, N-($C_1$-$C_4$)-alkylcarbamoyl, N,N-di-($C_1$-$C_4$)-alkylcarbamoyl, ($C_1$-$C_6$)-alkylcarbonyloxy, ($C_3$-$C_8$)-cycloalkyl, sulfamoyl, N-($C_1$-$C_4$)-alkylsulfamoyl, N,N-di-($C_1$-$C_4$)-alkylsulfamoyl; or wherein A is -$CR^5R^6$ and $R^5$ and $R^6$ are each independently selected from hydrogen, ($C_1$-$C_6$)-alkyl, ($C_3$-$C_7$)-cycloalkyl, aryl, or a substituent of the α-carbon atom of an α-amino acid, wherein the amino acid is a natural L-amino acid or its D-isomer.

B is -$CO_2H$, -$NH_2$, -$NHSO_2CF_3$, tetrazolyl, imidazolyl, 3-hydroxyisoxazolyl, -CONHCOR''', -CONHSOR''', $CONHSO_2R'''$, where R''' is aryl, heteroaryl, ($C_3$-$C_7$)-cycloalkyl, or ($C_1$-$C_4$)-alkyl, optionally monosubstituted by ($C_6$-$C_{12}$)-aryl, heteroaryl, OH, SH, ($C_1$-$C_4$)-alkyl, ($C_1$-$C_4$)-alkoxy, ($C_1$-$C_4$)-thioalkyl, ($C_1$-$C_4$)-sulfinyl, ($C_1$-$C_4$)-sulfonyl, $CF_3$, Cl, Br, F, I, NO2, -COOH, ($C_2$-$C_5$)-alkoxycarbonyl, $NH_2$, mono-($C_1$-$C_4$-alkyl)-amino, di-($C_1$-$C_4$-alkyl)-amino, or ($C_1$-$C_4$)-perfluoroalkyl; or wherein B is a $CO_2$-G carboxyl radical, where G is a radical of an alcohol G-OH in which G is selected from ($C_1$-$C_{20}$)-alkyl radical, ($C_3$-$C_8$) cycloalkyl radical, ($C_2$-$C_{20}$)-alkenyl radical, ($C_3$-$C_8$)-cycloalkenyl radical, retinyl radical, ($C_2$-$C_{20}$)-alkynyl radical, ($C_4$-$C_{20}$)-alkenynyl radical, where the alkenyl, cycloalkenyl, alkynyl, and alkenynyl radicals contain one or more multiple bonds; ($C_6$-$C_{16}$)-carbocyclic aryl radical, ($C_7$-$C_{16}$)-carbocyclic aralkyl radical, heteroaryl radical, or heteroaralkyl radical, wherein a heteroaryl

radical or heteroaryl moiety of a heteroaralkyl radical contains 5 or 6 ring atoms; and wherein radicals defined for G are substituted by one or more hydroxyl, halogen, cyano, trifluoromethyl, nitro, carboxyl, $(C_1-C_{12})$-alkyl, $(C_3-C_8)$-cycloalkyl, $(C_5-C_8)$-cycloalkenyl, $(C_6-C_{12})$-aryl, $(C_7-C_{16})$-aralkyl, $(C_2-C_{12})$-alkenyl, $(C_2-C_{12})$-alkynyl, $(C_1-C_{12})$-alkoxy, $(C_1-C_{12})$-alkoxy-$(C_1-C_{12})$-alkyl, $(C_1-C_{12})$-alkoxy-$(C_1-C_{12})$-alkoxy, $(C_6-C_{12})$-aryloxy, $(C_7-C_{16})$-aralkyloxy, $(C_1-C_8)$-hydroxyalkyl, $-O-[CH_2]_x-C_fH_{(2f+1-g)}-F_g$, $-OCF_2Cl$, $-OCF_2-CHFCl$, $(C_1-C_{12})$-alkylcarbonyl, $(C_3-C_8)$-cycloalkylcarbonyl, $(C_6-C_{12})$-arylcarbonyl, $(C_7-C_{16})$-aralkylcarbonyl, cinnamoyl, $(C_2-C_{12})$-alkenylcarbonyl, $(C_2-C_{12})$-alkynylcarbonyl, $(C_1-C_{12})$-alkoxycarbonyl, $(C_1-C_{12})$-alkoxy-$(C_1-C_{12})$-alkoxycarbonyl, $(C_6-C_{12})$-aryloxycarbonyl, $(C_7-C_{16})$-aralkoxycarbonyl, $(C_3-C_8)$-cycloalkoxycarbonyl, $(C_2-C_{12})$-alkenyloxycarbonyl, $(C_2-C_{12})$-alkynyloxycarbonyl, acyloxy, $(C_1-C_{12})$-alkoxycarbonyloxy, $(C_1-C_{12})$-alkoxy-$(C_1-C_{12})$-alkoxycarbonyloxy, $(C_6-C_{12})$-aryloxycarbonyloxy, $(C_7-C_{16})$ aralkyloxycarbonyloxy, $(C_3-C_8)$-cycloalkoxycarbonyloxy, $(C_2-C_{12})$-alkenyloxycarbonyloxy, $(C_2-C_{12})$-alkynyloxycarbonyloxy, carbamoyl, N-$(C_1-C_{12})$-alkylcarbamoyl, N.N-di$(C_1-C_{12})$-alkylcarbamoyl, N-$(C_3-C_8)$-cycloalkyl-carbamoyl, N-$(C_6-C_{16})$-arylcarbamoyl, N-$(C_7-C_{16})$-aralkylcarbamoyl, N-$(C_1-C_{10})$-alkyl-N-$(C_6-C_{16})$-arylcarbamoyl, N-$(C_1-C_{10})$-alkyl-N-$(C_7-C_{16})$-aralkylcarbamoyl, N-$((C_1-C_{10})$-alkoxy-$(C_1-C_{10})$-alkyl)-carbamoyl, N-$((C_6-C_{12})$-aryloxy-$(C_1-C_{10})$alkyl)-carbamoyl, N-$((C_7-C_{16})$-aralkyloxy-$(C_1-C_{10})$-alkyl)-carbamoyl, N-$(C_1-C_{10})$-alkyl-N-$((C_1-C_{10})$-alkoxy-$(C_1-C_{10})$-alkyl)-carbamoyl, N-$(C_1-C_{10})$-alkyl-N-$((C_6-C_{16})$-aryloxy-$(C_1-C_{10})$-alkyl)-carbamoyl, N-$(C_1-C_{10})$-alkyl-N-$((C_7-C_{16})$-aralkyloxy-$(C_1-C_{10})$-alkyl)-carbamoyl, carbamoyloxy, N-$(C_1-C_{12})$-alkylcarbamoyloxy, N.N-di-$(C_1-C_{12})$-alkylcarbamoyloxy, N-$(C_3-C_8)$-cycloalkylcarbamoyloxy, N-$(C_6-C_{12})$-arylcarbamoyloxy, N-$(C_7-C_{16})$-aralkylcarbamoyloxy, N-$(C_1-C_{10})$-alkyl-N-$(C_6-C_{12})$-arylcarbamoyloxy, N$(C_1-C_{10})$-alkyl-N-$(C_7-C_{16})$-aralkylcarbamoyloxy, N-$((C_1-C_{10})$-alkyl)-carbamoyloxy, N-$((C_6-C_{12})$-aryloxy-$(C_1-C_{10})$-alkyl)-carbamoyloxy, N-$((C_7-C_{16})$-aralkyloxy-$(C_1-C_{10})$-alkyl)-carbamoyloxy, N-$(C_1-C_{10})$-alkyl-N-$((C_1-C_{10})$-alkoxy-$(C_1-C_{10})$-alkyl)-carbamoyloxy, N-$(C_1-C_{10})$-alkyl-N-$((C_6-C_{12})$-aryloxy-$(C_1-C_{10})$-alkyl)-carbamoyloxy, N-$(C_1-C_{10})$-alkyl-N-$((C_7-C_{16})$-aralkyloxy-$(C_1-C_{10})$-alkyl)-carbamoyloxy, amino, $(C_1-C_{12})$-alkylamino, di-$(C_1-C_{12})$-alkylamino, $(C_3-C_8)$-cycloalkylamino, $(C_2-C_{12})$-alkenylamino, $(C_2-C_{12})$-alkynylamino, N-$(C_6-C_{12})$-arylamino, N-$(C-C_{11})$-aralkylamino, N-alkyl-aralkylamino, N-alkyl-arylamino, $(C_1-C_{12})$-alkoxyamino, $(C_1-C_{12})$-alkoxy-N-$(C_1-C_{10})$-alkylamino, $(C_1-C_{12})$-alkylcarbonylamino, $(C_3-C_8)$-cycloalkylcarbonylamino, $(C_6-C_{12})$ arylcarbonylamino, $(C_7-C_{16})$-aralkylcarbonylamino, $(C_1-C_{12})$-alkylcarbonyl-N-$(C_1-C_{10})$-alkylamino, $(C_3-C_8)$-cycloalkylcarbonyl-N-$(C_1-C_{10})$-alkylamino, $(C_6-C_{12})$-arylcarbonyl-N-$(C_1-C_{10})$alkylamino, $(C_7-C_{11})$-aralkylcarbonyl-N-$(C_1-C_{10})$-alkylamino, $(C_1-C_{12})$-alkylcarbonylamino-$(C_1-C_8)$-alkyl, $(C_3-C_8)$-cycloalkylcarbonylamino-$(C_1-C_8)$alkyl, $(C_6-C_{12})$-arylcarbonylamino-$(C_1-C_8)$-alkyl, $(C_7-C_{12})$-aralkylcarbonylamino$(C_1-C_8)$-alkyl, amino-$(C_1-C_{10})$-alkyl, N-$(C_1-C_{10})$ alkylamino-$(C_1-C_{10})$-alkyl, N.N-di-$(C_1-C_{10})$-alkylamino-$(C_1-C_{10})$-alkyl, $(C_3-C_8)$cycloalkylamino-$(C_1-C_{10})$-

alkyl, ($C_1$-$C_{12}$)-alkylmercapto, ($C_1$-$C_{12}$)-alkylsulfinyl, ($C_1$-$C_{12}$)-alkylsulfonyl, ($C_6$-$C_{16}$)-arylmercapto, ($C_6$-$C_{16}$)-arylsulfinyl, ($C_6$-$C_{12}$)-arylsulfonyl, ($C_7$-$C_{16}$)-aralkylmercapto, ($C_7$-$C_{16}$)-aralkylsulfinyl, ($C_7$-$C_{16}$)-aralkylsulfonyl, sulfamoyl, N-($C_1$-$C_{10}$)-alkylsulfamoyl, N.N-di($C_1$-$C_{10}$)-alkylsulfamoyl, ($C_3$-$C_8$)-cycloalkylsulfamoyl, N-($C_6$-$C_{12}$)-alkylsulfamoyl, N-($C_7$-$C_{16}$)-aralkylsulfamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_6$-$C_{12}$)-arylsulfamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_7$-$C_{16}$)-aralkylsulfamoyl, ($C_1$-$C_{10}$)-alkylsulfonamido, N-(($C_1$-$C_{10}$)-alkyl)-($C_1$-$C_{10}$)-alkylsulfonamido, ($C_7$-$C_{16}$)-aralkylsulfonamido, or N-(($C_1$-$C_{10}$)-alkyl-($C_7$-$C_{16}$)-aralkylsulfonamido; wherein radicals which are aryl or contain an aryl moiety, may be substituted on the aryl by one to five identical or different hydroxyl, halogen, cyano, trifluoromethyl, nitro, carboxyl, ($C_1$-$C_{12}$)-alkyl, ($C_3$-$C_8$)-cycloalkyl, ($C_6$-$C_{12}$)-aryl, ($C_7$-$C_{16}$)-aralkyl, ($C_1$-$C_{12}$)-alkoxy, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)alkyl, ($C_1$-$C_{12}$)-alkoxy-($C_1$ $C_{12}$)alkoxy, ($C_6$-$C_{12}$)-aryloxy, ($C_7$-$C_{16}$)-aralkyloxy, ($C_1$-$C_8$)-hydroxyalkyl, ($C_1$-$C_{12}$)-alkylcarbonyl, ($C_3$-$C_8$)-cycloalkyl-carbonyl, ($C_6$-$C_{12}$)-arylcarbonyl, ($C_7$-$C_{16}$) aralkylcarbonyl, ($C_1$-$C_{12}$)-alkoxycarbonyl, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)-alkoxycarbonyl, ($C_6$-$C_{12}$)-aryloxycarbonyl, ($C_7$-$C_{16}$)-aralkoxycarbonyl, ($C_3$-$C_8$)-cycloalkoxycarbonyl, ($C_2$-$C_{12}$)-alkenyloxycarbonyl, ($C_2$-$C_{12}$)-alkynyloxycarbonyl, ($C_1$-$C_{12}$)-alkylcarbonyloxy, ($C_3$-$C_8$)-cycloalkylcarbonyloxy, ($C_6$-$C_{12}$)-arylcarbonyloxy, ($C_7$-$C_{16}$)-aralkylcarbonyloxy, cinnamoyloxy, ($C_2$-$C_{12}$)-alkenylcarbonyloxy, ($C_2$-$C_{12}$)-alkynylcarbonyloxy, ($C_1$-$C_{12}$)-alkoxycarbonyloxy, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)-alkoxycarbonyloxy, ($C_6$-$C_{12}$)-aryloxycarbonyloxy, ($C_7$-$C_{16}$)-aralkyloxycarbonyloxy, ($C_3$-$C_8$)-cycloalkoxycarbonyloxy, ($C_2$-$C_{12}$)-alkenyloxycarbonyloxy, ($C_2$-$C_{12}$)-alkynyloxycarbonyloxy, carbamoyl, N-($C_1$-$C_{12}$)-alkylcarbamoyl, N.N-di-($C_1$-$C_{12}$)-alkylcarbamoyl, N-($C_3$-$C_8$)-cycloalkylcarbamoyl, N-($C_6$-$C_{12}$)-arylcarbamoyl, N-($C_7$-$C_{16}$)-aralkylcarbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_6$-$C_{12}$)-arylcarbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_7$-$C_{16}$)-aralkylcarbamoyl, N-(($C_1$-$C_{10}$)-alkoxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-(($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-(($C_1$-$C_{10}$)-alkoxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-(($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, carbamoyloxy, N-($C_1$-$C_{12}$)-alkylcarbamoyloxy, N.N-di-($C_1$-$C_{12}$)-alkylcarbamoyloxy, N-($C_3$-$C_8$)-cycloalkylcarbamoyloxy, N-($C_6$-$C_{12}$)-arylcarbamoyloxy, N-($C_7$-$C_{16}$)-aralkylcarbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-($C_6$-$C_{12}$)-arylcarbamoyloxy, N($C_1$-$C_{10}$)-alkyl-N-($C_7$-$C_{16}$)-aralkylcarbamoyloxy, N-(($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, N-(($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-(($C_1$-$C_{10}$)-alkoxy-($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-(($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyloxy, amino, ($C_1$-$C_{12}$)-alkylamino, di-($C_1$-$C_{12}$)-alkylamino, ($C_3$-$C_8$)-cycloalkylamino, ($C_3$-$C_{12}$)-alkenylamino, ($C_3$-$C_{12}$)-alkynylamino, N-($C_6$-$C_{12}$)-arylamino, N-($C_7$-$C_{11}$)-aralkylamino, N-alkylaralkylamino, N-alkyl-arylamino, ($C_1$-$C_{12}$)-alkoxyamino, ($C_1$-$C_{12}$)-alkoxy-N-($C_1$-$C_{10}$)-alkylamino, ($C_1$-$C_{12}$)-

alkylcarbonylamino, ($C_3$-$C_8$)-cycloalkylcarbonylamino, ($C_6$-$C_{12}$)-arylcarbonylamino, ($C_7$-$C_{16}$)-alkylcarbonylamino, ($C_1$-$C_{12}$)-alkylcarbonyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_3$-$C_8$)-cycloalkylcarbonyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_6$-$C_{12}$)-arylcarbonyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_7$-$C_{11}$)-aralkylcarbonyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_1$-$C_{12}$)-alkylcarbonylamino-($C_1$-$C_8$)-alkyl, ($C_3$-$C_8$)-cycloalkylcarbonylamino-($C_1$-$C_8$)-alkyl, ($C_6$-$C_{12}$)-arylcarbonylamino-($C_1$-$C_8$)-alkyl, ($C_7$-$C_{16}$)-aralkylcarbonylamino-($C_1$-$C_8$)-alkyl, amino-($C_1$-$C_{10}$)-alkyl, N-($C_1$-$C_{10}$)-alkylamino-($C_1$-$C_{10}$)alkyl, N,N-di-($C_1$-$C_{10}$)-alkylamino-($C_1$-$C_{10}$)-alkyl, ($C_3$-$C_8$)-cycloalkylamino-($C_1$-$C_{10}$)-alkyl, ($C_1$-$C_{12}$)-alkylmercapto, ($C_1$-$C_{12}$)-alkylsulfinyl, ($C_1$-$C_{12}$)-alkylsulfonyl, ($C_6$-$C_{12}$)-arylmercapto, ($C_6$-$C_{12}$)-arylsulfinyl, ($C_6$-$C_{12}$)-arylsulfonyl, ($C_7$-$C_{16}$)-aralkylmercapto, ($C_7$-$C_{16}$)-aralkylsulfinyl, or ($C_7$-$C_{16}$)-aralkylsulfonyl;

X is O or S;

Q is O, S, NR', or a bond;

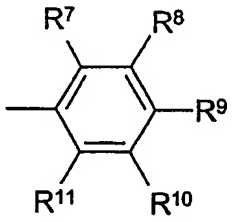
where, if Q is a bond, $R^4$ is halogen, nitrile, or trifluoromethyl;

or where, if Q is O, S, or NR', $R^4$ is hydrogen, ($C_1$-$C_{10}$)-alkyl radical, ($C_2$-$C_{10}$)-alkenyl radical, ($C_2$-$C_{10}$)-alkynyl radical, wherein alkenyl or alkynyl radical contains one or two C-C multiple bonds; unsubstituted fluoroalkyl radical of the formula $-[CH_2]_x-C_fH_{(2f+1-g)}-F_g$, ($C_1$-$C_8$)-alkoxy-($C_1$-$C_6$)-alkyl radical, ($C_1$-$C_6$)-alkoxy-($C_1$-$C_4$)-alkoxy-($C_1$-$C_4$)-alkyl radical, aryl radical, heteroaryl radical, ($C_7$-$C_{11}$)-aralkyl radical, or a radical of the formula Z

$$-[CH_2]_v-[O]_w-[CH_2]_t-E \qquad (Z)$$

where

E is a heteroaryl radical, a ($C_3$-$C_8$)-cycloalkyl radical, or a phenyl radical of the formula F




(F)

v is 0-6,

w is 0 or 1,

t is 0-3, and

$R^7$, $R^8$, $R^9$, $R^{10}$, and $R^{11}$ are identical or different and are hydrogen, halogen, cyano, nitro, trifluoromethyl, ($C_1$-$C_6$)-alkyl, ($C_3$-$C_8$)-cycloalkyl, ($C_1$-$C_6$)-alkoxy, $-O-[CH_2]_x-C_fH_{(2f+1-g)}-F_g$, $-OCF_2-Cl$, $-O-CF_2-CHFCl$, ($C_1$-$C_6$)-alkylmercapto, ($C_1$-$C_6$)-hydroxyalkyl, ($C_1$-$C_6$)-alkoxy-($C_1$-

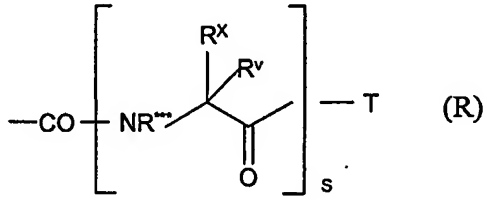
$C_6$)-alkoxy, ($C_1$-$C_6$)-alkoxy-($C_1$-$C_6$)-alkyl, ($C_1$-$C_6$)-alkylsulfinyl, ($C_1$-$C_6$)-alkylsulfonyl, ($C_1$-$C_6$)-alkylcarbonyl, ($C_1$-$C_8$)-alkoxycarbonyl, carbamoyl, N-($C_1$-$C_8$)-alkylcarbamoyl, N,N-di-($C_1$-$C_8$)-alkylcarbamoyl, or ($C_7$-$C_{11}$)-aralkylcarbamoyl, optionally substituted by fluorine, chlorine, bromine, trifluoromethyl, ($C_1$-$C_6$)-alkoxy, N-($C_3$-$C_8$)-cycloalkylcarbamoyl, N-($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_4$)-alkylcarbamoyl, ($C_1$-$C_6$)-alkylcarbonyloxy, phenyl, benzyl, phenoxy, benzyloxy, $NR^yR^z$ wherein $R^y$ and $R^z$ are independently selected from hydrogen, ($C_1$-$C_{12}$)-alkyl, ($C_1$-$C_8$)-alkoxy-($C_1$-$C_8$)-alkyl, ($C_7$-$C_{12}$)-aralkoxy-($C_1$-$C_8$)-alkyl, ($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_8$)-alkyl, ($C_3$-$C_{10}$)-cycloalkyl, ($C_3$-$C_{12}$)-alkenyl, ($C_3$-$C_{12}$)-alkynyl, ($C_6$-$C_{12}$)-aryl, ($C_7$-$C_{11}$)-aralkyl, ($C_1$-$C_{12}$)-alkoxy, ($C_7$-$C_{12}$)aralkoxy, ($C_1$-$C_{12}$)-alkylcarbonyl, ($C_3$-$C_8$)-cycloalkylcarbonyl, ($C_6$-$C_{12}$) arylcarbonyl, ($C_7$-$C_{16}$)-aralkylcarbonyl; or further wherein $R^y$ and $R^z$ together are -[CH2 ]$_h$ , in which a $CH_2$ group can be replaced by O, S, N-($C_1$-$C_4$)-alkylcarbonylimino, or N-($C_1$-$C_4$)-alkoxycarbonylimino; phenylmercapto, phenylsulfonyl, phenylsulfinyl, sulfamoyl, N-($C_1$-$C_8$)-alkylsulfamoyl, or N, N-di-($C_1$-$C_8$)-alkylsulfamoyl; or alternatively $R^7$ and $R^8$, $R^8$ and $R^9$, $R^9$ and $R^{10}$, or $R^{10}$ and $R^{11}$, together are a chain selected from -[$CH_2$]$_n$- or -CH=CH-CH=CH-, where a $CH_2$ group of the chain is optionally replaced by O, S, SO, $SO_2$, or $NR^Y$; and n is 3, 4, or 5; and if E is a heteroaryl radical, said radical can carry 1-3 substituents selected from those defined for $R^7$-$R^{11}$, or if E is a cycloalkyl radical, the radical can carry one substituent selected from those defined for $R^7$-$R^{11}$;

or where, if Q is NR', $R^4$ is alternatively R", where R' and R" are identical or different and are hydrogen, ($C_6$-$C_{12}$)-aryl, ($C_7$-$C_{11}$)-aralkyl, ($C_1$-$C_8$)-alkyl, ($C_1$-$C_8$)-alkoxy-($C_1$-$C_8$)-alkyl, ($C_7$-$C_{12}$)-aralkoxy-($C_1$-$C_8$)-alkyl, ($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_8$)-alkyl, ($C_1$-$C_{10}$)-alkylcarbonyl, optionally substituted ($C_7$-$C_{16}$)-aralkylcarbonyl, or optionally substituted $C_6$-$C_{12}$)-arylcarbonyl; or R' and R" together are -[$CH_2$]$_h$, in which a $CH_2$ group can be replaced by O, S, N-acylimino, or N-($C_1$-$C_{10}$)-alkoxycarbonylimino, and h is 3 to 7.

Y is N or $CR^3$;

$R^1$, $R^2$ and $R^3$ are identical or different and are hydrogen, hydroxyl, halogen, cyano, trifluoromethyl, nitro, carboxyl, ($C_1$-$C_{20}$)-alkyl, ($C_3$-$C_8$)-cycloalkyl, ($C_3$-$C_8$)cycloalkyl-($C_1$-$C_{12}$)-alkyl, ($C_3$-$C_8$)-cycloalkoxy, ($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_{12}$)-alkoxy, ($C_3$-$C_8$)-cycloalkyloxy-($C_1$-$C_{12}$)-alkyl, ($C_3$-$C_8$)-cycloalkyloxy-($C_1$-$C_{12}$)-alkoxy, ($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_8$)-alkyl-($C_1$-$C_6$)-alkoxy, ($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_8$)-alkoxy-($C_1$-$C_6$)-alkyl, ($C_3$-$C_8$)-cycloalkyloxy-($C_1$-$C_8$)-alkoxy-($C_1$-$C_6$)-alkyl, ($C_3$-$C_8$)-cycloalkoxy-($C_1$-$C_8$)-alkoxy-($C_1$-$C_8$)-alkoxy, ($C_6$-$C_{12}$)-aryl, ($C_7$-$C_{16}$)-aralkyl, ($C_7$-$C_{16}$)-aralkenyl, ($C_7$-$C_{16}$)-aralkynyl, ($C_2$-$C_{20}$)-alkenyl, ($C_2$-$C_{20}$)-alkynyl, ($C_1$-$C_{20}$)-alkoxy, ($C_2$-$C_{20}$)-alkenyloxy, ($C_2$-$C_{20}$)-alkynyloxy, retinyloxy, ($C_1$-$C_{20}$)-alkoxy-($C_1$-$C_{12}$)-alkyl, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)-alkoxy, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_8$)-alkoxy-($C_1$-$C_8$)-alkyl, ($C_6$-

$C_{12}$)-aryloxy, ($C_7$-$C_{16}$)-aralkyloxy, ($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_6$)-alkoxy, ($C_7$-$C_{16}$)-aralkoxy-($C_1$-$C_6$)-alkoxy, ($C_1$-$C_{16}$)-hydroxyalkyl, ($C_6$-$C_{16}$)-aryloxy-($C_1$-$C_8$)-alkyl, ($C_7$-$C_{16}$)-aralkoxy-($C_1$-$C_8$)-alkyl, ($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_8$)-alkoxy-($C_1$-$C_6$)-alkyl, ($C_7$-$C_{12}$)-aralkyloxy-($C_1$-$C_8$)-alkoxy-($C_1$-$C_6$)-alkyl, ($C_2$-$C_{20}$)-alkenyloxy-($C_1$-$C_6$)-alkyl, ($C_2$-$C_{20}$)-alkynyloxy-($C_1$-$C_6$)-alkyl, retinyloxy-($C_1$-$C_6$)-alkyl, -O-$[CH_2]_xCfH_{(2f+1-g)}F_g$, $-OCF_2Cl$, $-OCF_2-CHFCl$, ($C_1$-$C_{20}$)-alkylcarbonyl, ($C_3$-$C_8$)-cycloalkylcarbonyl, ($C_6$-$C_{12}$)-arylcarbonyl, ($C_7$-$C_{16}$)-aralkylcarbonyl, cinnamoyl, ($C_2$-$C_{20}$)-alkenylcarbonyl, ($C_2$-$C_{20}$)-alkynylcarbonyl, ($C_1$-$C_{20}$)-alkoxycarbonyl, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)-alkoxycarbonyl, ($C_6$-$C_{12}$)-aryloxycarbonyl, ($C_7$-$C_{16}$)-aralkoxycarbonyl, ($C_3$-$C_8$)-cycloalkoxycarbonyl, ($C_2$-$C_{20}$)-alkenyloxycarbonyl, retinyloxycarbonyl, ($C_2$-$C_{20}$)-alkynyloxycarbonyl, ($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_6$)-alkoxycarbonyl, ($C_7$-$C_{16}$)-aralkoxy-($C_1$-$C_6$)-alkoxycarbonyl, ($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_6$)-alkoxycarbonyl, ($C_3$-$C_8$)-cycloalkoxy-($C_1$-$C_6$)-alkoxycarbonyl, ($C_1$-$C_{12}$)-alkylcarbonyloxy, ($C_3$-$C_8$)-cycloalkylcarbonyloxy, ($C_6$-$C_{12}$)-arylcarbonyloxy, ($C_7$-$C_{16}$)-aralkylcarbonyloxy, cinnamoyloxy, ($C_2$-$C_{12}$)-alkenylcarbonyloxy, ($C_2$-$C_{12}$)-alkynylcarbonyloxy, ($C_1$-$C_{12}$)-alkoxycarbonyloxy, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)-alkoxycarbonyloxy, ($C_6$-$C_{12}$)-aryloxycarbonyloxy, ($C_7$-$C_{16}$)-aralkyloxycarbonyloxy, ($C_3$-$C_8$)-cycloalkoxycarbonyloxy, ($C_2$-$C_{12}$)-alkenyloxycarbonyloxy, ($C_2$-$C_{12}$)-alkynyloxycarbonyloxy, carbamoyl, N-($C_1$-$C_{12}$)-alkylcarbamoyl, N,N-di-($C_1$-$C_{12}$)-alkylcarbamoyl, N-($C_3$-$C_8$)-cycloalkylcarbamoyl, N,N-dicyclo-($C_3$-$C_8$)-alkylcarbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_3$-$C_8$)-cycloalkylcarbamoyl, N-(($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_6$)-alkyl)-carbamoyl, N-($C_1$-$C_6$)-alkyl-N-(($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_6$)-alkyl)-carbamoyl, N-(+)-dehydroabietylcarbamoyl, N-($C_1$-$C_6$)-alkyl-N-(+)-dehydroabietylcarbamoyl, N-($C_6$-$C_{12}$)-arylcarbamoyl, N-($C_7$-$C_{16}$)-aralkylcarbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_6$-$C_{16}$)-arylcarbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_7$-$C_{16}$)-aralkylcarbamoyl, N-(($C_1$-$C_{18}$)-alkoxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-(($C_6$-$C_{16}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-(($C_1$-$C_{10}$)-alkoxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-(($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl; $CON(CH_2)_h$, in which a $CH_2$ group can be replaced by O, S, N-($C_1$-$C_8$)-alkylimino, N-($C_3$-$C_8$)-cycloalkylimino, N-($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_4$)-alkylimino, N-($C_6$-$C_{12}$)-arylimino, N-($C_7$-$C_{16}$)-aralkylimino, N-($C_1$-$C_4$)-alkoxy-($C_1$-$C_6$)-alkylimino, and h is from 3 to 7; a carbamoyl radical of the formula R




(R)

in which

$R^x$ and $R^y$ are each independently selected from hydrogen, $(C_1-C_6)$-alkyl, $(C_3-C_7)$-cycloalkyl, aryl, or the substituent of an α-carbon of an α-amino acid, to which the L- and D-amino acids belong,

s is 1-5,

T is OH, or NR*R**, and R*, R** and R*** are identical or different and are selected from hydrogen, $(C_6-C_{12})$-aryl, $(C_7-C_{11})$-aralkyl, $(C_1-C_8)$-alkyl, $(C_3-C_8)$-cycloalkyl, (+)-dehydroabietyl, $(C_1-C_8)$-alkoxy-$(C_1-C_8)$-alkyl, $(C_7-C_{12})$-aralkoxy-$(C_1-C_8)$-alkyl, $(C_6-C_{12})$-aryloxy-$(C_1-C_8)$-alkyl, $(C_1-C_{10})$-alkanoyl, optionally substituted $(C_7-C_{16})$-aralkanoyl, optionally substituted $(C_6-C_{12})$-aroyl; or R* and R** together are $-[CH_2]_h-$, in which a $CH_2$ group can be replaced by O, S, SO, $SO_2$, N-acylamino, N-$(C_1-C_{10})$-alkoxycarbonylimino, N-$(C_1-C_8)$-alkylimino, N-$(C_3-C_8)$-cycloalkylimino, N-$(C_3-C_8)$-cycloalkyl-$(C_1-C_4)$-alkylimino, N-$(C_6-C_{12})$-arylimino, N-$(C_7-C_{16})$-aralkylimino, N-$(C_1-C_4)$-alkoxy-$(C_1-C_6)$-alkylimino, and h is from 3 to 7;

carbamoyloxy, N-$(C_1-C_{12})$-alkylcarbamoyloxy, N,N-di-$(C_1-C_{12})$-alkylcarbamoyloxy, N-$(C_3-C_8)$-cycloalkylcarbamoyloxy, N-$(C_6-C_{12})$-arylcarbamoyloxy, N-$(C_7-c_{16})$-aralkylcarbamoyloxy, N-$(C_1-C_{10})$-alkyl-N-$(C_6-C_{12})$-arylcarbamoyloxy, N-$(C_1-C_{10})$-alkyl-N-$(C_7-C_{16})$-aralkylcarbamoyloxy, N-$((C_1-C_{10})$-alkyl)-carbamoyloxy, N-$((C_6-C_{12})$-aryloxy-$(C_1-C_{10})$-alkyl)-carbamoyloxy, N-$((C_7-C_{16})$-aralkyloxy-$(C_1-C_{10})$-alkyl)-carbamoyloxy, N-$(C_1-C_{10})$-alkyl-N-$((C_1-C_{10})$-alkoxy-$(C_1-C_{10})$-alkyl)-carbamoyloxy, N-$(C_1-C_{10})$-alkyl-N-$((C_6-C_{12})$-aryloxy-$(C_1-C_{10})$-alkyl)-carbamoyloxy, N-$(C_1-C_{10})$-alkyl-N-$((C_7-C_{16})$-aralkyloxy-$(C_1-C_{10})$-alkyl)-carbamoyloxyamino, $(C_1-C_{12})$-alkylamino, di-$(C_1-C_{12})$-alkylamino, $(C_3-C_8)$-cycloalkylamino, $(C_3-C_{12})$-alkenylamino, $(C_3-C_{12})$-alkynylamino, N-$(C_6-C_{12})$-arylamino, N-$(C_7-C_{11})$-aralkylamino, N-alkyl-aralkylamino, N-alkyl-arylamino, $(C_1-C_{12})$-alkoxyamino, $(C_1-C_{12})$-alkoxy-N-$(C_1-C_{10})$-alkylamino, $(C_1-C_{12})$-alkanoylamino, $(C_3-C_8)$-cycloalkanoylamino, $(C_6-C_{12})$-aroylamino, $(C_7-C_{16})$-aralkanoylamino, $(C_1-C_{12})$-alkanoyl-N-$(C_1-C_{10})$-alkylamino, $(C_3-C_8)$-cycloalkanoyl-N-$(C_1-C_{10})$-alkylamino, $(C_6-C_{12})$-aroyl-N-$(C_1-C_{10})$-alkylamino, $(C_7-C_{11})$-aralkanoyl-N-$(C_1-C_{10})$-alkylamino, $(C_1-C_{12})$-alkanoylamino-$(C_1-C_8)$-alkyl, $(C_3-C_8)$-cycloalkanoylamino-$(C_1-C_8)$-alkyl, $(C_6-C_{12})$-aroylamino-$(C_1-C_8)$-alkyl, $(C_7-C_{16})$-aralkanoylamino-$(C_1-C_8)$-alkyl, amino-$(C_1-C_{10})$-alkyl, N-$(C_1-C_{10})$-alkylamino-$(C_1-C_{10})$-alkyl, N,N-di$(C_1-C_{10})$-alkylamino-$(C_1-C_{10})$-alkyl, $(C_3-C_8)$-cycloalkylamino$(C_1-C_{10})$-alkyl, $(C_1-C_{20})$-alkylmercapto, $(C_1-C_{20})$-alkylsulfinyl, $(C_1-C_{20})$-alkylsulfonyl, $(C_6-C_{12})$-arylmercapto, $(C_6-C_{12})$-arylsulfinyl, $(C_6-C_{12})$-arylsulfonyl, $(C_7-C_{16})$-aralkylmercapto, $(C_7-C_{16})$-aralkylsulfinyl, $(C_7-C_{16})$-aralkylsulfonyl, $(C_1-C_{12})$-alkylmercapto-$(C_1-C_6)$-alkyl, $(C_1-C_{12})$-alkylsulfinyl-$(C_1-C_6)$-alkyl, $(C_1-C_{12})$-alkylsulfonyl-$(C_1-C_6)$-alkyl, $(C_6-C_{12})$-arylmercapto-$(C_1-C_6)$-alkyl, $(C_6-C_{12})$-arylsulfinyl-$(C_1-C_6)$-alkyl, $(C_6-C_{12})$-arylsulfonyl-$(C_1-C_6)$-alkyl, $(C_7-C_{16})$-aralkylmercapto-$(C_1-C_6)$-alkyl, $(C_7-C_{16})$-aralkylsulfinyl-$(C_1-C_6)$-alkyl, $(C_7-C_{16})$-aralkylsulfonyl-$(C_1-C_6)$-alkyl, sulfamoyl, N-$(C_1-C_{10})$-alkylsulfamoyl, N,N-di-$(C_1-C_{10})$-alkylsulfamoyl, $(C_3-C_8)$-

cycloalkylsulfamoyl, N-($C_6$-$C_{12}$)-arylsulfamoyl, N-($C_7$-$C_{16}$)-aralkylsulfamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_6$-$C_{12}$)-arylsulfamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_7$-$C_{16}$)-aralkylsulfamoyl, ($C_1$-$C_{10}$)-alkylsulfonamido, N-(($C_1$-$C_{10}$)-alkyl)-($C_1$-$C_{10}$)-alkylsulfonamido, ($C_7$-$C_{16}$)-aralkylsulfonamido, and N-(($C_1$-$C_{10}$)-alkyl-($C_7$-$C_{16}$)-aralkylsulfonamido; where an aryl radical may be substituted by 1 to 5 substituents selected from hydroxyl, halogen, cyano, trifluoromethyl, nitro, carboxyl, ($C_2$-$C_{16}$)-alkyl, ($C_3$-$C_8$)-cycloalkyl, ($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_{12}$)-alkyl, ($C_3$-$C_8$)-cycloalkoxy, ($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_{12}$)-alkoxy, ($C_3$-$C_8$)-cycloalkyloxy-($C_1$-$C_{12}$)-alkyl, ($C_3$-$C_8$)-cycloalkyloxy-($C_1$-$C_{12}$)-alkoxy, ($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_8$)-alkyl-($C_1$-$C_6$)-alkoxy, ($C_3$-$C_8$)-cycloalkyl($C_1$-$C_8$)-alkoxy-($C_1$-$C_6$)-alkyl, ($C_3$-$C_8$)-cycloalkyloxy-($C_1$-$C_8$)-alkoxy-($C_1$-$C_6$)-alkyl, ($C_3$-$C_8$)-cycloalkoxy-($C_1$-$C_8$)-alkoxy-($C_1$-$C_8$)-alkoxy, ($C_6$-$C_{12}$)-aryl, ($C_7$-$C_{16}$)-aralkyl, ($C_2$-$C_{16}$)-alkenyl, ($C_2$-$C_{12}$)-alkynyl, ($C_1$-$C_{16}$)-alkoxy, ($C_1$-$C_{16}$)-alkenyloxy, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)-alkyl, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)-alkoxy, ($C_1$-$C_{12}$)-alkoxy($C_1$-$C_8$)-alkoxy-($C_1$-$C_8$)-alkyl, ($C_6$-$C_{12}$)-aryloxy, ($C_7$-$C_{16}$)-aralkyloxy, ($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_6$)-alkoxy, ($C_7$-$C_{16}$)-aralkoxy-($C_1$-$C_6$)-alkoxy, ($C_1$-$C_8$)-hydroxyalkyl, ($C_6$-$C_{16}$)-aryloxy-($C_1$-$C_8$)-alkyl, ($C_7$-$C_{16}$)-aralkoxy-($C_1$-$C_8$)-alkyl, ($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_8$)-alkoxy-($C_1$-$C_6$)-alkyl, ($C_7$-$C_{12}$)-aralkyloxy-($C_1$-$C_8$)-alkoxy-($C_1$-$C_6$)-alkyl, -O-$[CH_2]_xC_fH_{(2f+1-g)}F_g$, -$OCF_2Cl$, -$OCF_2$-CHFCl, ($C_1$-$C_{12}$)-alkylcarbonyl, ($C_3$-$C_8$)-cycloalkylcarbonyl, ($C_6$-$C_{12}$)-arylcarbonyl, ($C_7$-$C_{16}$)-aralkylcarbonyl, ($C_1$-$C_{12}$)-alkoxycarbonyl, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)-alkoxycarbonyl, ($C_6$-$C_{12}$)-aryloxycarbonyl, ($C_7$-$C_{16}$)-aralkoxycarbonyl, ($C_3$-$C_8$)-cycloalkoxycarbonyl, ($C_2$-$C_{12}$)-alkenyloxycarbonyl, ($C_2$-$C_{12}$)-alkynyloxycarbonyl, ($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_6$)-alkoxycarbonyl, ($C_7$-$C_{16}$)-aralkoxy-($C_1$-$C_6$)-alkoxycarbonyl, ($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_6$)-alkoxycarbonyl, ($C_3$-$C_8$)-cycloalkoxy-($C_1$-$C_6$)-alkoxycarbonyl, ($C_1$-$C_{12}$)-alkylcarbonyloxy, ($C_3$-$C_8$)-cycloalkylcarbonyloxy, ($C_6$-$C_{12}$)-arylcarbonyloxy, ($C_7$-$C_{16}$)-aralkylcarbonyloxy, cinnamoyloxy, ($C_2$-$C_{12}$)-alkenylcarbonyloxy, ($C_2$-$C_{12}$)-alkynylcarbonyloxy, ($C_1$-$C_{12}$)-alkoxycarbonyloxy, ($C_1$-$C_{12}$)-alkoxy-($C_1$-$C_{12}$)-alkoxycarbonyloxy, ($C_6$-$C_{12}$)-aryloxycarbonyloxy, ($C_7$-$C_{16}$)-aralkyloxycarbonyloxy, ($C_3$-$C_8$)-cycloalkoxycarbonyloxy, ($C_2$-$C_{12}$)-alkenyloxycarbonyloxy, ($C_2$-$C_{12}$)-alkynyloxycarbonyloxy, carbamoyl, N-($C_1$-$C_{12}$)-alkylcarbamoyl, N,N-di($C_1$-$C_{12}$)-alkylcarbamoyl, N-($C_3$-$C_8$)-cycloalkylcarbamoyl, N,N-dicyclo-($C_3$-$C_8$)-alkylcarbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_3$-$C_8$)-cycloalkylcarbamoyl, N-(($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_6$)-alkyl)carbamoyl, N-($C_1$-$C_6$)-alkyl-N-(($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_6$)-alkyl)carbamoyl, N-(+)-dehydroabietylcarbamoyl, N-($C_1$-$C_6$)-alkyl-N-(+)-dehydroabietylcarbamoyl, N-($C_6$-$C_{12}$)-arylcarbamoyl, N-($C_7$-$C_{16}$)-aralkylcarbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_6$-$C_{16}$)-arylcarbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-($C_7$-$C_{16}$)-aralkylcarbamoyl, N-(($C_1$-$C_{16}$)-alkoxy-($C_1$-$C_{10}$)-alkyl)carbamoyl, N-(($C_6$-$C_{16}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)carbamoyl, N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)carbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-(($C_1$-$C_{10}$)-alkoxy-($C_1$-$C_{10}$)-alkyl)carbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-(($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)carbamoyl, N-($C_1$-$C_{10}$)-alkyl-N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)-carbamoyl, $CON(CH_2)_h$, in which a

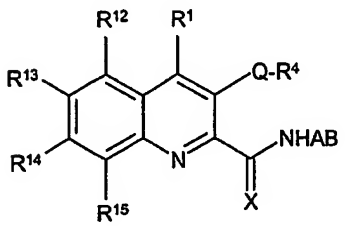
$CH_2$ group can be replaced by, O, S, N-($C_1$-$C_8$)-alkylimino, N-($C_3$-$C_8$)-cycloalkylimino, N-($C_3$-$C_8$)-cycloalkyl-($C_1$-$C_4$)-alkylimino, N-($C_6$-$C_{12}$)-arylimino, N-($C_7$-$C_{16}$)-aralkylimino, N-($C_1$-$C_4$)-alkoxy-($C_1$-$C_6$)-alkylimino, and h is from 3 to 7; carbamoyloxy, N-($C_1$-$C_{12}$)-alkylcarbamoyloxy, N,N-di-($C_1$-$C_{12}$)-alkylcarbamoyloxy, N-($C_3$-$C_8$)-cycloalkylcarbamoyloxy, N-($C_6$-$C_{16}$)-arylcarbamoyloxy, N-($C_7$-$C_{16}$)-aralkylcarbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-($C_6$-$C_{12}$)-arylcarbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-($C_7$-$C_{16}$)-aralkylcarbamoyloxy, N-(($C_1$-$C_{10}$)-alkyl)carbamoyloxy, N-(($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)carbamoyloxy, N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)carbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-(($C_1$-$C_{10}$)-alkoxy-($C_1$-$C_{10}$)-alkyl)carbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-(($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_{10}$)-alkyl)carbamoyloxy, N-($C_1$-$C_{10}$)-alkyl-N-(($C_7$-$C_{16}$)-aralkyloxy-($C_1$-$C_{10}$)-alkyl)carbamoyloxy, amino, ($C_1$-$C_{12}$)-alkylamino, di-($C_1$-$C_{12}$)-alkylamino, ($C_3$-$C_8$)-cycloalkylamino, ($C_3$-$C_{12}$)-alkenylamino, ($C_3$-$C_{12}$)-alkynylamino, N-($C_6$-$C_{12}$)-arylamino, N-($C_7$-$C_{11}$)-aralkylamino, N-alkyl-aralkylamino, N-alkyl-arylamino, ($C_1$-$C_{12}$)-alkoxyamino, ($C_1$-$C_{12}$)-alkoxy-N-($C_1$-$C_{10}$)-alkylamino, ($C_1$-$C_{12}$)-alkanoylamino, ($C_3$-$C_8$)-cycloalkanoylamino, ($C_6$-$C_{12}$)-aroylamino, ($C_7$-$C_{16}$)-aralkanoylamino, ($C_1$-$C_{12}$)-alkanoyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_3$-$C_8$)-cycloalkanoyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_6$-$C_{12}$)-aroyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_7$-$C_{11}$)-aralkanoyl-N-($C_1$-$C_{10}$)-alkylamino, ($C_1$-$C_{12}$)-alkanoylamino-($C_1$-$C_8$)-alkyl, ($C_3$-$C_8$)-cycloalkanoylamino-($C_1$-$C_8$)-alkyl, ($C_6$-$C_{12}$)-aroylamino- ($C_1$-$C_8$)-alkyl, ($C_7$-$C_{16}$)-aralkanoylamino-($C_1$-$C_8$)-alkyl, amino-($C_1$-$C_{10}$)-alkyl, N-($C_1$-$C_{10}$)-alkylamino-($C_1$-$C_{10}$)-alkyl, N,N-di-($C_1$-$C_{10}$)-alkylamino-($C_1$-$C_{10}$)-alkyl, ($C_3$-$C_8$)-cycloalkylamino-($C_1$-$C_{10}$)-alkyl, ($C_1$-$C_{12}$)-alkylmercapto, ($C_1$-$C_{12}$)-alkylsulfinyl, ($C_1$-$C_{12}$)-alkylsulfonyl, ($C_6$-$C_{16}$)-arylmercapto, ($C_6$-$C_{16}$)-arylsulfinyl, ($C_6$-$C_{16}$)-arylsulfonyl, ($C_7$-$C_{16}$)-aralkylmercapto, ($C_7$-$C_{16}$)-aralkylsulfinyl, or ($C_7$-$C_{16}$)-aralkylsulfonyl;

or wherein $R^1$ and $R^2$, or $R^2$ and $R^3$ form a chain $[CH_2]_o$, which is saturated or unsaturated by a C=C double bond, in which 1 or 2 $CH_2$ groups are optionally replaced by O, S, SO, $SO_2$, or NR', and R' is hydrogen, ($C_6$-$C_{12}$)-aryl, ($C_1$-$C_8$)-alkyl, ($C_1$-$C_8$)-alkoxy-($C_1$-$C_8$)-alkyl, ($C_7$-$C_{12}$)-aralkoxy-($C_1$-$C_8$)-alkyl, ($C_6$-$C_{12}$)-aryloxy-($C_1$-$C_8$)-alkyl, ($C_1$-$C_{10}$)-alkanoyl, optionally substituted ($C_7$-$C_{16}$)-aralkanoyl, or optionally substituted (C6-C12)-aroyl; and o is 3, 4 or 5;

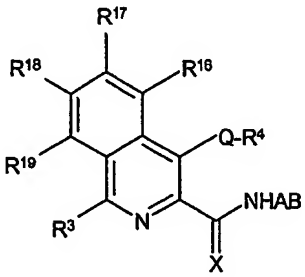
or wherein the radicals $R^1$ and $R^2$, or $R^2$ and $R^3$, together with the pyridine or pyridazine carrying them, form a 5,6,7,8-tetrahydroisoquinoline ring, a 5,6,7,8-tetrahydroquinoline ring, or a 5,6,7,8-tetrahydrocinnoline ring;

or wherein $R^1$ and $R^2$, or $R^2$ and $R^3$ form a carbocyclic or heterocyclic 5- or 6-membered aromatic ring;

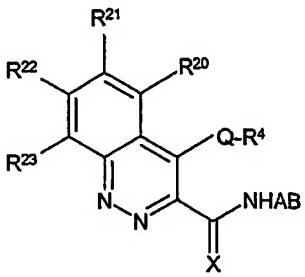
or where $R^1$ and $R^2$, or $R^2$ and $R^3$, together with the pyridine or pyridazine carrying them, form an optionally substituted heterocyclic ring systems selected from thienopyridines, furanopyridines, pyridopyridines, pyrimidinopyridines, imidazopyridines, thiazolopyridines, oxazolopyridines, quinoline, isoquinoline, and cinnoline; where quinoline, isoquinoline or cinnoline preferably satisfy the formulae Ia, Ib and Ic:




(Ia)



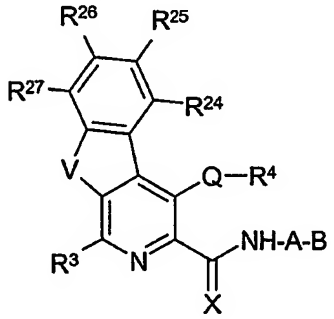

(Ib)




(Ic)

and the substituents $R^{12}$ to $R^{23}$ in each case independently of each other have the meaning of $R^1$, $R^2$ and $R^3$;

or wherein the radicals $R^1$ and $R^2$, together with the pyridine carrying them, form a compound of Formula Id:




(Id)

where V is S, O, or $NR^k$, and $R^k$ is selected from hydrogen, $(C_1$-$C_6)$-alkyl, aryl, or benzyl; where an aryl radical may be optionally substituted by 1 to 5 substituents as defined above; and

$R^{24}$, $R^{25}$, $R^{26}$, and $R^{27}$ in each case independently of each other have the meaning of $R^1$, $R^2$ and $R^3$;

f is 1 to 8;
g is 0 or 1 to (2f+1);
x is 0 to 3; and
h is 3 to 7;

including the physiologically active salts and prodrugs derived therefrom.

[0084] Exemplary compounds according to Formula (I) are described in European Patent Nos. EP0650960 and EP0650961. All compounds listed in EP0650960 and EP0650961, in particular, those listed in the compound claims and the final products of the working examples, are hereby incorporated into the present application by reference herein. Exemplary compounds of Formula (I) include, but are not limited to, [(3-Hydroxy-pyridine-2-carbonyl)-amino]-acetic acid (Compound G) and [(3-methoxy-pyridine-2-carbonyl)-amino]-acetic acid (Compound P).

[0085] Additionally, exemplary compounds according to Formula (I) are described in U.S. Patent No. 5,658,933. All compounds listed in U.S. Patent No. 5,658,933, in particular, those listed in the compound claims and the final products of the working examples, are hereby incorporated into the present application by reference herein. Exemplary compounds of Formula (I) include, but are not limited to, 3-methoxypyridine-2-carboxylic acid N-(((hexadecyloxy)-carbonyl)-methyl)-amide hydrochloride, 3-methoxypyridine-2-carboxylic acid N-(((1-octyloxy)-carbonyl)-methyl)-amide, 3-methoxypyridine-2-carboxylic acid N-(((hexyloxy)-carbonyl)-methyl)-amide, 3-methoxypyridine-2-carboxylic acid N-(((butyloxy)-carbonyl)-methyl)-amide, 3-methoxypyridine-2-carboxylic acid N-(((2-nonyloxy)-carbonyl)-methyl)-amide racemate, 3-methoxypyridine-2-carboxylic acid N-(((heptyloxy)-carbonyl)-methyl)-amide, 3-benzyloxypyridine-2-carboxylic acid N-(((octyloxy)-carbonyl)-methyl)-amide, 3-benzyloxypyridine-2-carboxylic acid N-(((butyloxy)-carbonyl)-methyl)-amide, 5-(((3-(1-butyloxy)-propyl)-amino)-carbonyl)-3-methoxypyridine-2-carboxylic acid N-((benzyloxycarbonyl)-methyl)-amide, 5-(((3-(1-butyloxy)-propyl)-amino)-carbonyl)-3-methoxypyridine-2-carboxylic acid N-(((1-butyloxy)-carbonyl)-methyl)-amide, and 5-(((3-lauryloxy)-propyl)amino)-carbonyl)-3-methoxypyridine-2-carboxylic acid N-(((benzyloxy)-carbonyl)-methyl)-amide.

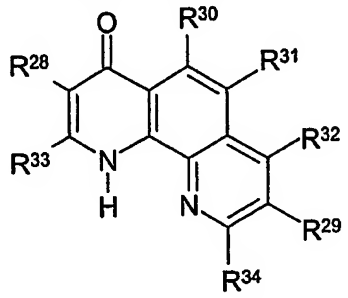
[0086] Additional compounds acording to Formula (I) are substituted heterocyclic carboxyamides described in U.S. Patent No. 5,620,995; 3-hydroxypyridine-2-carboxamidoesters described in U.S. Patent No. 6,020,350; sulfonamidocarbonylpyridine-2-carboxamides described in U.S. Patent No. 5,607,954; and sulfonamidocarbonyl-pyridine-2-carboxamides and sulfonamidocarbonyl-pyridine-2-carboxamide esters described in U.S. Patent Nos. 5,610,172 and 5,620,996. All compounds listed in these patents, in particular, those compounds listed in the compound claims and the final products of the working examples, are hereby incorporated into the present application by reference herein.

[0087] Exemplary compounds according to Formula (Ia) are described in U.S. Patent Nos. 5,719,164 and 5,726,305. All compounds listed in the foregoing patents, in particular, those listed in the compound claims and the final products of the working examples, are hereby incorporated into the present application by reference herein. Exemplary compounds of Formula (1a) include, but are not limited to, N-((3-hydroxy-6-isopropoxy-quinoline-2-carbonyl)-amino)-acetic acid (Compound H), N-((6-(1-butyloxy)-3-hydroxyquinolin-2-yl)-carbonyl)-glycine, [(3-hydroxy-6-trifluoromethoxy-quinoline-2-carbonyl)-amino]-acetic acid (Compound I), N-((6-chloro-3-hydroxyquinolin-2-yl)-carbonyl)-glycine, N-((7-chloro-3-hydroxyquinolin-2-yl)-carbonyl)-glycine, and [(6-chloro-3-hydroxy-quinoline-2-carbonyl)-amino]-acetic acid (Compound O).

[0088] Exemplary compounds according to Formula (Ib) are described in U.S. Patent No. 6,093,730. All compounds listed in U.S. Patent No. 6,093,730, in particular, those listed in the compound claims and the final products of the working examples, are hereby incorporated into the present application by reference herein. Exemplary compounds of Formula (1b) include, but are not limited to, N-((1-chloro-4-hydroxy-7-(2-propyloxy) isoquinolin-3-yl)-carbonyl)-glycine, N-((1-chloro-4-hydroxy-6-(2-propyloxy) isoquinolin-3-yl)-carbonyl)-glycine, N-((1-chloro-4-hydroxy-isoquinoline-3-carbonyl)-amino)-acetic acid (Compound B), N-((1-chloro-4-hydroxy-7-methoxyisoquinolin-3-yl)-carbonyl)-glycine, N-((1-chloro-4-hydroxy-6-methoxyisoquinolin-3-yl)-carbonyl)-glycine, N-((7-butyloxy)-1-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine, N-((6-benzyloxy-1-chloro-4-hydroxy-isoquinoline-3-carbonyl)-amino)-acetic acid (Compound J), ((7-benzyloxy-1-chloro-4-hydroxy-isoquinoline-3-carbonyl)-amino)-acetic acid methyl ester (Compound K), N-((7-benzyloxy-1-chloro-4-hydroxy-isoquinoline-3-carbonyl)-amino)-acetic acid (Compound L), N-((8-chloro-4-hydroxyisoquinolin-3-yl)-carbonyl)-glycine, N-((7-butoxy-4-hydroxy-isoquinoline-3-carbonyl)-amino)-acetic acid (Compound M).

[0089] Additionally, compounds related to Formula (I) that can also be used in the methods of the invention include, but are not limited to, 6-cyclohexyl-1-hydroxy-4-methyl-1H-pyridin-2-one (Compound N), 7-(4-methyl-piperazin-1-ylmethyl)-5-phenylsulfanylmethyl-quinolin-8-ol (Compound D), 4-nitro-quinolin-8-ol (Compound E), and 5-butoxymethyl-quinolin-8-ol (Compound F). Further, the invention provides additional exemplary compounds wherein, e.g., position A and B together may be, e.g., hexanoic acid, cyanomethyl, 2-aminoethyl, benzoic acid, 1H-benzoimidazol-2-ylmethyl, etc.

[0090] In other embodiments, compounds used in the methods of the invention are selected from a compound of the formula (II)



(II)

where

$R^{28}$ is hydrogen, nitro, amino, cyano, halogen, ($C_1$-$C_4$)-alkyl, carboxy or a metabolically labile ester derivative thereof; ($C_1$-$C_4$)-alkylamino, di-($C_1$-$C_4$)-alkylamino, ($C_1$-$C_6$)-alkoxycarbonyl, ($C_2$-$C_4$)-alkanoyl, hydroxy-($C_1$-$C_4$)-alkyl, carbamoyl, N-($C_1$-$C_4$)-alkylcarbamoyl, ($C_1$-$C_4$)-alkylthio, ($C_1$-$C_4$)-alkylsulfinyl, ($C_1$-$C_4$)-alkylsulfonyl, phenylthio, phenylsulfinyl, phenylsulfonyl, said phenyl or phenyl groups being optionally substituted with 1 to 4 identical or different halogen, ($C_1$-$C_4$)-alkyoxy, ($C_1$-$C_4$)-alkyl, cyano, hydroxy, trifluoromethyl, fluoro-($C_1$-$C_4$)-alkylthio, fluoro-($C_1$-$C_4$)-alkylsulfinyl, fluoro-($C_1$-$C_4$)-alkylsulfonyl, ($C_1$-$C_4$)-alkoxy-($C_2$-$C_4$)-alkoxycarbonyl, N,N-di-[($C_1$-$C_4$)-alkyl]carbamoyl-($C_1$-$C_4$)-alkoxycarbonyl, ($C_1$-$C_4$)-alkylamino-($C_2$-$C_4$)-alkoxycarbonyl, di-($C_1$-$C_4$)-alkylamino-($C_2$-$C_4$)-alkoxycarbonyl, ($C_1$-$C_4$)-alkoxy-($C_2$-$C_4$)-alkoxy-($C_2$-$C_4$)-alkoxycarbonyl, ($C_2$-$C_4$)-alkanoyloxy-$C_1$-$C_4$)-alkyl, or N-[amino-($C_2$-$C_8$)-alkyl]-carbamoyl;

$R^{29}$ is hydrogen, hydroxy, amino, cyano, halogen, ($C_1$-$C_4$)-alkyl, carboxy or metabolically labile ester derivative thereof, ($C_1$-$C_4$)-alkylamino, di-($C_1$-$C_4$)-alkylamino, ($C_1$-$C_6$)-alkoxycarbonyl, ($C_2$-$C_4$)-alkanoyl, ($C_1$-$C_4$)-alkoxy, carboxy-($C_1$-$C_4$)-alkoxy, ($C_1$-$C_4$)-alkoxycarbonyl-($C_1$-$C_4$)-alkoxy, carbamoyl, N-($C_1$-$C_8$)-alkylcarbamoyl, N,N-di-($C_1$-$C_8$)-alkylcarbamoyl, N-[amino-($C_2$-$C_8$)-alkyl)-carbamoyl, N-[($C_1$-$C_4$)-alkylamino-($C_1$-$C_8$)-alkyl]-carbamoyl, N-[di-($C_1$-$C_4$)-alkylamino-($C_1$-$C_8$)-alkyl)]-carbamoyl, N-cyclohexylcarbamoyl, N-[cyclopentyl]-carbamoyl, N-($C_1$-$C_4$)-alkylcyclohexylcarbamoyl, N-($C_1$-$C_4$)-alkylcyclopentylcarbamoyl, N-phenylcarbamoyl, N-($C_1$-$C_4$)-alkyl-N-phenylcarbamoyl, N,N-diphenylcarbamoyl, N-[phenyl-($C_1$-$C_4$)-alkyl]-carbamoyl, N-($C_1$-$C_4$)-alkyl-N-[phenyl-($C_1$-$C_4$)-alkyl]-carbamoyl, or N,N-di-[phenyl-($C_1$-$C_4$)-alkyl]-carbamoyl, said phenyl or phenyl groups being optionally substituted with 1 to 4 identical or different halogen, ($C_1$-$C_4$)-alkyoxy, ($C_1$-$C_4$)-alkyl, cyano, hydroxy, trifluoromethyl, N-[($C_2$-$C_4$)-alkanoyl]-carbamoyl, N-[($C_1$-$C_4$)-alkoxycarbonyl]-carbamoyl, N-[fluoro-($C_2$-$C_6$)-alkyl]-carbamoyl, N,N-[fluoro-($C_2$-$C_6$)-alkyl]-N-($C_1$-$C_4$)-alkylcarbamoyl, N,N-[di-fluoro-($C_2$-$C_6$)-alkyl]carbamoyl, pyrrolidin-1-ylcarbonyl, piperidinocarbonyl, piperazin-1-ylcarbonyl, morpholinocarbonyl, wherein the

heterocyclic group, is optionally substituted with 1 to 4, ($C_1$-$C_4$)-alkyl, benzyl , 1,2,3,4-tetrahydro-isoquinolin-2-ylcarbonyl, N,N-[di-($C_1$-$C_4$)-alkyl]-thiocarbamoyl, N-($C_2$-$C_4$)-alkanoylamino, or N-[($C_1$-$C_4$)-alkoxycarbonyl]-amino;

$R^{30}$ is hydrogen, ($C_1$-$C_4$)-alkyl, ($C_2$-$C_4$)-alkoxy, halo, nitro, hydroxy, fluoro-(1-4C)alkyl, or pyridinyl;

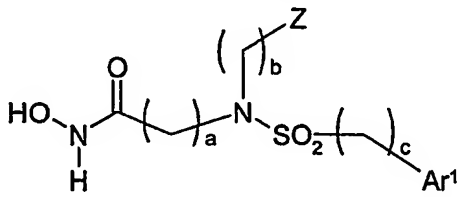
$R^{31}$ is hydrogen, ($C_1$-$C_4$)-alkyl, ($C_2$-$C_4$)-alkoxy, halo, nitro, hydroxy, fluoro-($C_1$-$C_4$)-alkyl, pyridinyl, or methoxy;

$R^{32}$ is hydrogen, hydroxy, amino, ($C_1$-$C_4$)-alkylamino, di-($C_1$-$C_4$)-alkylamino, halo, ($C_1$-$C_4$)-alkoxy-($C_2$-$C_4$)-alkoxy, fluoro-($C_1$-$C_6$)-alkoxy, pyrrolidin-1-yl, piperidino, piperazin-1-yl, or morpholino, wherein the heterocyclic group is optionally substituted with 1 to 4 identical or different ($C_1$-$C_4$)-alkyl or benzyl; and

$R^{33}$ and $R^{34}$ are individually selected from hydrogen, ($C_1$-$C_4$)-alkyl, and ($C_1$-$C_4$)-alkoxy; including pharmaceutically-acceptable salts and pro-drugs derived therefrom.

[0091] Exemplary compounds of Formula (II) are described in U.S. Patent Nos. 5,916,898 and 6,200,974, and International Publication No. WO 99/21860. All compounds listed in the foregoing patents and publication, in particular, those listed in the compound claims and the final products of the working examples, are hereby incorporated into the present application by reference herein. Exemplary compounds of Formula (II) include 4-oxo-1,4-dihydro-[1,10]phenanthroline-3-carboxylic acid (Compound A) (see, e.g., Seki et al. (1974) Chem Abstracts 81:424, No. 21), 3-carboxy-5-hydroxy-4-oxo-3,4-dihydro-1,10-phenanthroline, 3-carboxy-5-methoxy-4-oxo-3,4-dihydro-1,10-phenanthroline, 5-methoxy-4-oxo-1,4-dihydro-[1,10]phenanthroline-3-carboxylic acid ethyl ester, 5-methoxy-4-oxo-1,4-dihydro-[1,10]phenanthroline-3-carboxylic acid (Compound Q), and 3-carboxy-8-hydroxy-4-oxo-3,4-dihydro-1,10-phenanthroline.

[0092] In other embodiments, compounds used in the methods of the invention are selected from a compound of the formula (III)




(III)

or pharmaceutically acceptable salts thereof, wherein:

a is an integer from 1 to 4;

b is an integer from 0 to 4;

c is an integer from 0 to 4;

Z is selected from the group consisting of ($C_3$-$C_{10}$) cycloalkyl, ($C_3$-$C_{10}$) cycloalkyl independently substituted with one or more $Y^1$, 3-10 membered heterocycloalkyl and 3-10 membered heterocycloalkyl independently substituted with one or more $Y^1$; ($C_5$-$C_{20}$) aryl, ($C_5$-$C_{20}$) aryl independently substituted with one or more $Y^1$, 5-20 membered heteroaryl and 5-20 membered heteroaryl independently substituted with one or more $Y^1$;

$Ar^1$ is selected from the group consisting of ($C_5$-$C_{20}$) aryl, ($C_5$-$C_{20}$) aryl independently substituted with one or more $Y^2$, 5-20 membered heteroaryl and 5-20 membered heteroaryl independently substituted with one or more $Y^2$;

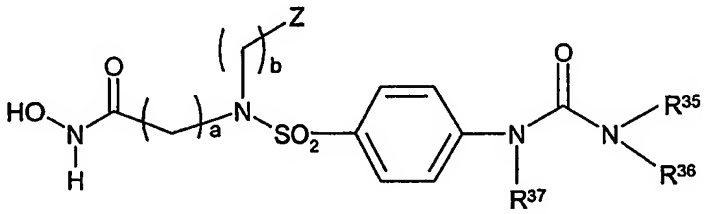
each $Y^1$ is independently selected from the group consisting of a lipophilic functional group, ($C_5$-$C_{20}$) aryl, ($C_6$-$C_{26}$) alkaryl, 5-20 membered heteroaryl and 6-26 membered alk-heteroaryl;

each $Y^2$ is independently selected from the group consisting of -R', -OR', -OR", -SR', -SR", -NR'R', -$NO_2$, -CN, -halogen, -trihalomethyl, trihalomethoxy, -C(O)R', -C(O)OR', -C(O)NR'R', -C(O)NR'OR', -C(NR'R')=NOR', -NR'-C(O)R', -$SO_2$R', -$SO_2$R", -NR'-$SO_2$-R', -NR'-C(O)-NR'R', tetrazol-5-yl, -NR'-C(O)-OR', -C(NR'R')=NR', -S(O)-R', -S(O)-R", and -NR'-C(S)-NR'R'; and

each R' is independently selected from the group consisting of -H, ($C_1$-$C_8$) alkyl, ($C_2$-$C_8$) alkenyl, and ($C_2$-$C_8$) alkynyl; and

each R" is independently selected from the group consisting of ($C_5$-$C_{20}$) aryl and ($C_5$-$C_{20}$) aryl independently substituted with one or more -OR', -SR', -NR'R', -$NO_2$, -CN, halogen or trihalomethyl groups,

or wherein c is 0 and $Ar^1$ is an N' substituted urea-aryl, the compound has the structural formula (IIIa):



(IIIa)

or pharmaceutically acceptable salts thereof, wherein:

a, b, and Z are as defined above; and

$R^{35}$ and $R^{36}$ are each independently selected from the group consisting of hydrogen, ($C_1$-$C_8$) alkyl, ($C_2$-$C_8$) alkenyl, ($C_2$-$C_8$) alkynyl, ($C_3$-$C_{10}$) cycloalkyl, ($C_5$-$C_{20}$) aryl, ($C_5$-$C_{20}$) substituted

aryl, ($C_6$-$C_{26}$) alkaryl, ($C_6$-$C_{26}$) substituted alkaryl, 5-20 membered heteroaryl, 5-20 membered substituted heteroaryl, 6-26 membered alk-heteroaryl, and 6-26 membered substituted alk-heteroaryl; and

$R^{37}$ is independently selected from the group consisting of hydrogen, ($C_1$-$C_8$) alkyl, ($C_2$-$C_8$) alkenyl, and ($C_2$-$C_8$) alkynyl.

[0093] Exemplary compounds of Formula (III) are described in International Publication No. WO 00/50390. All compounds listed in WO 00/50390, in particular, those listed in the compound claims and the final products of the working examples, are hereby incorporated into the present application by reference herein. Exemplary compounds of Formula (III) include 3-{[4-(3,3-dibenzyl-ureido)-benzenesulfonyl]-[2-(4-methoxy-phenyl)-ethyl]-amino}-N-hydroxy-propionamide (Compound C), 3-{{4-[3-(4-chloro-phenyl)-ureido]-benzenesulfonyl}-[2-(4-methoxy-phenyl)-ethyl]-amino}-N-hydroxy-propionamide, and 3-{{4-[3-(1,2-diphenyl-ethyl)-ureido]-benzenesulfonyl}-[2-(4-methoxy-phenyl)-ethyl]-amino}-N-hydroxy-propionamide.

[0094] Based on the common mechanism of action of the 2-oxoglutarate dioxygenase family members, such as dependence on $Fe^{2+}$ and 2-oxoglutarate for activity, in certain aspects the invention is directed to use of compounds, including the compounds described herein, to inhibit HIF$\alpha$ hydroxylation and thus stabilize HIF$\alpha$ in an oxygen-independent manner. Further, the examples and figures of the present invention demonstrate that application of such compounds stabilize HIF$\alpha$ and subsequently induce HIF-regulated gene products *in vitro* and *in vivo*. In specific embodiments, these compounds are used to produce a specific benefit in the prevention and treatment of ischemic and hypoxic conditions.

[0095] The methods of the present invention stabilize HIF$\alpha$ in a dose-dependent manner in cells grown in a normoxic environment. Although different cell types show different levels of HIF$\alpha$ in the presence of a compound of the invention, all of the cell lines tested showed some level of HIF$\alpha$ stabilization. The level of HIF$\alpha$ in untreated cells is usually low to undetectable.

[0096] Stabilization of HIF$\alpha$ leads to HIF-dependent gene expression *in vitro* and *in vivo*, including genes encoding angiogenic factors such as VEGF, Flt-1, EG-VEGF, PAI-1, adrenomedullin, and Cyr61. Thus, the ability to stabilize HIF$\alpha$ has potential benefits in the induction of angiogenesis and prevention of tissue damage due to ischemia and hypoxia. For example, transgenic mice expressing constitutively active HIF-1$\alpha$ in the epidermis show enhanced expression of each VEGF isoform and a significant increase in dermal capillaries.

Unlike overexpression of one VEGF isoform alone, the hypervascularity induced by HIFα shows no edema, inflammation, or vascular leakage. (See, Elson et al. (2001) Genes Dev 15:2520-2532; Detmar et al. (1998) J Invest Derm 111:1–6; Larcher et al. (1998) Oncogene 17:303-311; and Thurston et al. (1999) Science 286:2511–2514.) Therefore, in certain aspects, methods of the invention can be used to induce therapeutic angiogenesis, which involves the development of collateral blood vessels to revascularize ischemic tissues.

[0097] Additionally, the methods of the invention produce a dose-dependent decrease in oxygen consumption in cells without any affect on cell viability. Stable HIF complexes activate expression of proteins involved in glucose uptake and utilization, such as glucose transporter (GluT)-1 and GluT-3; aldolase-A, enolase-1, hexokinase-1 and -2, and phosphofructokinase-L and -C. The reduction in oxygen consumption associated with HIFα stabilization is potentially due to a shift in cellular metabolism from aerobic to anaerobic energy production. The present methods can thus be applied to generate energy under low oxygen conditions, beneficial in ischemic and hypoxic conditions such as, for example, peripheral arterial disease, DVT, angina pectoris, pulmonary embolism, stroke, and myocardial infarction. Methods of increasing glucose uptake and utilization by cells of the body, generally applicable to the treatment of other conditions, e.g., diabetes, are also provided.

[0098] The invention further provides methods for increasing oxygen-carrying capacity by inducing erythropoiesis, and facilitating iron transport and utilization. Specifically, methods of the invention increase expression of erythropoietin (EPO), a naturally occurring hormone that stimulates the production of red blood cells. (See, e.g., commonly owned, copending U.S. Patent Application Serial No. _____, entitled "Methods for Increasing Endogenous Erythropoietin (EPO)," filed of even date, and incorporated herein by reference in its entirety.) Methods for increasing expression of enzymes and proteins involved in iron uptake, transport, and processing are specifically contemplated. Such enzymes and proteins include, but are not limited to, transferrin and transferrin receptor, which together facilitate iron transport to and uptake by, e.g., erythroid tissue; and ceruloplasmin, a ferroxidase required to oxidize ferrous iron to ferric iron. As transferrin can only bind and transport ferric iron, ceruloplasmin is important for supply of iron to tissues. The ability of the methods of the invention to increase both endogenous erythropoietin and transport and utilization of iron provides specific advantage in oxygen delivery in both normoxic and hypoxic environments.

[0099] In one aspect, the invention includes methods that provide neuroprotective benefits, e.g., by stabilizing HIFα. For example, both VEGF and EPO have been shown to be neuroprotective. (See, e.g., Jin et al. (2000) Proc Natl Acad Sci USA. 97:10242-10247; Bocker-

Meffert et al. (2002) Invest Ophthalmol Vis Sci 43:2021-2026; Buemi et al. (2002) Clin Sci (Lond) 103:275-282; and Siren et al. (2001) Proc Natl Acad Sci USA 98:4044-4049.) EPO also facilitates recovery from spinal cord injuries and provides neuroprotective benefits when induced prior to an ischemic event. (See, e.g., Gorio et al. (2002) Proc Natl Acad Sci USA 99:9450-9455; and Dawson (2002) Lancet 359:96-97.) As the methods of the invention increase expression of neuroprotective factors such as VEGF and EPO, the methods provide neuroprotective benefit that can be applied to treatment, pretreatment, or prevention of conditions including, e.g., diabetic neuropathy, stroke, neurodegenerative disease, trauma, injury, e.g., concussions, spinal cord injuries, etc., or prior to surgical procedures, e.g., wherein cerebral ischemic reperfusion injury may result.

**[00100]** Hypoxic preconditioning has been shown to effectively protect against subsequent acute ischemic insult. As the primary effect of hypoxia is stabilization of HIF$\alpha$ and subsequent activation of HIF-regulated genes, the methods of the invention will mimic hypoxic preconditioning in a normoxic environment. For example, the methods may be used prior to surgery, wherein ischemic-reperfusion injury may be expected to produce deleterious results in the patient. Such preventive therapy, when applied prior to an ischemic event, can be provided at any time point prior to the event, in a single or repeated dose format.

**[00101]** The methods of the invention also coordinately upregulate genes involved in oxidative stress and vascular tone. Such genes include, e.g., inducible nitric oxide synthase (iNOS), and heme oxygenase 1. Production of iNOS has also been associated with the beneficial effects of hypoxic preconditioning in several animal models. (See, e.g., Serracino-Inglott et al. (2002) BMC Gastroenterol 2:22-27; Kuntscher et al. (2002) Br J Plast Surg 55:430-433.) Significantly, blocking iNOS activity attenuates but does not abrogate the beneficial effects of preconditioning, whereas nonspecifically blocking protein production completely abrogates the benefits of preconditioning. (Wang et al. (2002) Cardiovasc Res 56:33-42.) This suggests that iNOS is an important component of the physiological response to preconditioning, but is not the only factor. As the methods of the invention coordinately regulate various factors, including iNOS, involved in hypoxic response, the methods of the invention will more accurately replicate the beneficial effects of hypoxic preconditioning.

Methods of Using the Compounds of the Invention

**[00102]** The present invention provides methods of inhibiting HIF$\alpha$ hydroxylation, thereby stabilizing HIF and activating HIF-regulated gene expression. The methods can be applied to the prevention, pretreatment, or treatment of conditions associated with HIF including ischemic and hypoxic conditions. Such conditions include, for example, myocardial infarction,

liver ischemia, renal ischemia, and stroke; peripheral vascular disorders, ulcers, burns, and chronic wounds; pulmonary embolism; and ischemic-reperfusion injury, including, for example, ischemic-reperfusion injury associated with surgery and organ transplantation. In one embodiment, the present invention provides methods of stabilizing HIFα before, during, or immediately after ischemia or hypoxia, particularly in association with myocardial infarction, stroke, or renal ischemic-reperfusion injury.

[00103] In one aspect, the invention provides methods for treating various ischemic and hypoxic conditions, in particular, using the compounds described herein. In one embodiment, the methods of the invention produce therapeutic benefit when administered following ischemia or hypoxia. For example, the methods of the invention produce a dramatic decrease in morbidity and mortality following myocardial infarction, and a significant improvement in heart architecture and performance. Further, the methods of the invention improve liver function when administered following hepatic toxic-ischemic injury. Hypoxia is a significant component of liver disease, especially in chronic liver disease associated with hepatotoxic compounds such as ethanol. Additionally, expression of genes known to be induced by HIFα, e.g., nitric oxide synthase and glucose transporter-1, is increased in alcoholic liver disease. (See, e.g., Areel et al. (1997) Hepatology 25:920-926; Strubelt (1984) Fundam Appl Toxicol 4:144-151; Sato (1983) Pharmacol Biochem Behav 18 (Suppl 1):443-447; Nanji et al. (1995) Am J Pathol 146:329-334; and Morio et al. (2001) Toxicol Appl Pharmacol 172:44-51.)

[00104] Therefore, the present invention provides methods of treating conditions associated with ischemia or hypoxia, the method comprising administering a therapeutically effective amount of a compound or a pharmaceutically acceptable salt thereof, alone or in combination with a pharmaceutically acceptable excipient, to a subject. In one embodiment, the compound is administered immediately following a condition producing acute ischemia, e.g., myocardial infarction, pulmonary embolism, intestinal infarction, ischemic stroke, and renal ischemic-reperfusion injury. In another embodiment, the compound is administered to a patient diagnosed with a condition associated with the development of chronic ischemia, e.g., cardiac cirrhosis, macular degeneration, pulmonary embolism, acute respiratory failure, neonatal respiratory distress syndrome, and congestive heart failure. In yet another embodiment, the compound is administered immediately after a trauma or injury.

[00105] In another aspect, the invention provides methods for treating a patient at risk of developing an ischemic or hypoxic condition, e.g., individuals at high risk for atherosclerosis, etc., using the compounds described herein. Risk factors for atherosclerosis include, e.g., hyperlipidemia, cigarette smoking, hypertension, diabetes mellitus,

hyperinsulinemia, and abdominal obesity. Therefore, the present invention provides methods of preventing ischemic tissue injury, the method comprising administering a therapeutically effective amount of a compound or a pharmaceutically acceptable salt thereof, alone or in combination with a pharmaceutically acceptable excipient, to a patient in need. In one embodiment, the compound can be administered based on predisposing conditions, e.g., hypertension, diabetes, occlusive arterial disease, chronic venous insufficiency, Raynaud's disease, chronic skin ulcers, cirrhosis, congestive heart failure, and systemic sclerosis.

[00106] In one specific embodiment, the methods are used to increase vascularization and/or granulation tissue formation in damaged tissue, wounds, and ulcers. For example, compounds of the invention have been shown to be effective in stimulating granulation tissue formation in wound healing. Granulation tissue contains newly formed, leaky blood vessels and a provisional stroma of plasma proteins, such as fibrinogen and plasma fibronectin. Release of growth factors from inflammatory cells, platelets, and activated endothelium, stimulates fibroblast and endothelial cell migration and proliferation within the granulation tissue. Ulceration can occur if vascularization or neuronal stimulation is impaired. The methods of the invention are effective at promoting granulation tissue formation. Thus, the invention provides methods for treating a patient having tissue damage due to, e.g., an infarct, having wounds induced by, e.g., trauma or injury, or having chronic wounds or ulcers produced as a consequence of a disorder, e.g., diabetes. The method comprises administering a therapeutically effective amount of a compound or a pharmaceutically acceptable salt thereof, alone or in combination with a pharmaceutically acceptable excipient, to a patient in need.

[00107] In another aspect, the invention provides methods of using the compounds to pretreat a subject to decrease or prevent the development of tissue damage associated with ischemia or hypoxia. The methods of the invention produce therapeutic benefit when administered immediately before a condition involving ischemia or hypoxia. For example, application of the methods of the invention prior to induction of myocardial infarction shows statistically significant improvement in heart architecture and performance. Further, the methods of the invention produce therapeutic benefit when administered immediately before and during ischemic-reperfusion injury, significantly reducing diagnostic parameters associated with renal failure.

[00108] Therefore, the invention provides methods of pretreating a subject to decrease or prevent the tissue damage associated with ischemia or hypoxia, the method comprising administering a therapeutically effective amount of a compound or a pharmaceutically

acceptable salt thereof, alone or in combination with a pharmaceutically acceptable excipient, to a patient with a history of ischemic disorders, e.g., myocardial infarctions, or having symptoms of impending ischemia, e.g.,angina pectoris. In another embodiment, the compound can be administered based on physical parameters implicating possible ischemia, e.g., individuals placed under general anesthesia or temporarily working at high altitudes. In yet another embodiment, the compounds may be used in organ transplants to pretreat organ donors and to maintain organs removed from the body prior to implantation in the recipient.

[00109] Previous studies have shown that certain compounds used in the methods of the present invention are effective inhibitors of procollagen prolyl 4-hydroxylase. While it is recognized that recovery from an initial infarct or wound requires connective tissue deposition within the necrotic region, the present invention demonstrates no adverse affects of treatment with respect to scar formation. Thus, based on the benefits provided by certain compounds of the invention on treatment and prevention of hypoxic tissue damage and fibrosis, the present invention contemplates a "dual-therapy" approach to treatment or prevention of conditions involving ischemia or hypoxia, including ischemia or hypoxia associated with subsequent reactive fibrosis, e.g., myocardial infarction and resultant congestive heart failure. The method may use one compound that inhibits more than one 2-oxoglutarate dioxygenase enzyme, e.g., HIF prolyl hydroxylase and procollagen prolyl 4-hydroxylase, with either the same specificity or with different specificities. Alternatively, the method may use a combination of compounds wherein each compound specifically inhibits only one 2-oxoglutarate dioxygenase enzyme, e.g., one compound specifically inhibits HIF prolyl hydroxylase and a second compound specifically inhibits procollagen prolyl 4-hydroxylase.

[00110] In one aspect, a compound of the invention inhibits one or more 2-oxoglutarate dioxygenase enzymes. In one embodiment, the compound inhibits at least two 2-oxoglutarate dioxygenase family members, e.g., HIF prolyl hydroxylase and HIF asparagine-hydroxylase (FIH-1), with either the same specificity or with differential specificity. In another embodiment, the compound is specific for one 2-oxoglutarate dioxygenase, e.g., HIF prolyl hydroxylase, and shows little to no specificity for other family members.

[00111] The compounds can be administered in combination with various other therapeutic approaches. In one embodiment, the compound is administered with another 2-oxoglutarate dioxygenase inhibitor, wherein the two compounds have differential specificity for individual 2-oxoglutarate dioxygenase family members. The two compounds may be administered at the same time as a ratio of one relative to the other. Determination of

a ratio appropriate to a given course of treatment or a particular subject is within the level of skill in the art. Alternatively, the two compounds may be administered consecutively during a treatment time course, e.g., following myocardial infarction. In a particular embodiment, one compound specifically inhibits HIF prolyl hydroxylase enzyme activity, and a second compound specifically inhibits procollagen prolyl 4-hydroxylase enzyme activity. In another specific embodiment, one compound specifically inhibits HIF prolyl hydroxylase enzyme activity, and a second compound specifically inhibits HIF asparaginyl-hydroxylase enzyme activity. In another embodiment, the compound is administered with another therapeutic agent having a different mode of action, e.g., an ACE inhibitor (ACEI), angiotensin-II receptor blocker (ARB), statin, diuretic, digoxin, carnitine, etc.

Pharmaceutical Formulations And Routes Of Administration

[00112] The compositions of the present invention can be delivered directly or in pharmaceutical compositions along with suitable carriers or excipients, as is well known in the art. Present methods of treatment can comprise administration of an effective amount of a compound of the invention to a subject having or at risk for an ischemic condition, e.g., congestive heart failure, atherosclerosis, etc. In a preferred embodiment, the subject is a mammalian subject, and in a most preferred embodiment, the subject is a human subject. Preferred routes of administration include oral and transdermal delivery mechanisms.

[00113] An effective amount of such agents can readily be determined by routine experimentation, as can the most effective and convenient route of administration and the most appropriate formulation. Various formulations and drug delivery systems are available and selection of an appropriate formulation is within the level of skill in the art. (See, e.g., Gennaro, ed. (1995) Remington's Pharmaceutical Sciences, *supra*; and Hardman, Limbird, and Gilman, eds. (2001) The Pharmacological Basis of Therapeutics, *supra*.)

[00114] Suitable routes of administration may, for example, include oral, rectal, transmucosal, nasal, or intestinal administration and parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections. The agent or composition thereof may be administered in a local rather than a systemic manner. For example, a suitable agent can be delivered via injection or in a targeted drug delivery system, such as a depot or sustained release formulation.

[00115] The pharmaceutical compositions of the present invention may be manufactured by any of the methods well-known in the art, such as by conventional mixing,

dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes. As noted above, the compositions of the present invention can include one or more physiologically acceptable carriers such as excipients and auxiliaries that facilitate processing of active molecules into preparations for pharmaceutical use.

[00116] Proper formulation is dependent upon the route of administration chosen. For injection, for example, the composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For transmucosal or nasal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject. The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

[00117] Pharmaceutical preparations for oral use can be obtained as solid excipients, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

[00118] Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

[00119] Pharmaceutical preparations for oral administration include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as

glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.

[00120] In one embodiment, the compounds of the present invention can be administered transdermally, such as through a skin patch, or topically. In one aspect, the transdermal or topical formulations of the present invention can additionally comprise one or multiple penetration enhancers or other effectors, including agents that enhance migration of the delivered compound. Transdermal or topical administration could be preferred, for example, in situations in which location specific delivery is desired.

[00121] For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide, or any other suitable gas. In the case of a pressurized aerosol, the appropriate dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, for example, gelatin, for use in an inhaler or insufflator may be formulated. These typically contain a powder mix of the compound and a suitable powder base such as lactose or starch.

[00122] Compositions formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion, can be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Formulations for parenteral administration include aqueous solutions or other compositions in water-soluble form.

[00123] Suspensions of the active compounds may also be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil and synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents that increase the solubility of the

compounds to allow for the preparation of highly concentrated solutions. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

[00124] As mentioned above, the compositions of the present invention may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example, subcutaneous or intramuscular) or by intramuscular injection. Thus, for example, the present compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

[00125] Suitable carriers for the hydrophobic molecules of the invention are well-known in the art and include co-solvent systems comprising, for example, benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. The co-solvent system may be the VPD co-solvent system. VPD is a solution of 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant polysorbate 80, and 65% w/v polyethylene glycol 300, made up to volume in absolute ethanol. The VPD co-solvent system (VPD:5W) consists of VPD diluted 1:1 with a 5% dextrose in water solution. This co-solvent system is effective in dissolving hydrophobic compounds and produces low toxicity upon systemic administration. Naturally, the proportions of a co-solvent system may be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components may be varied. For example, other low-toxicity nonpolar surfactants may be used instead of polysorbate 80, the fraction size of polyethylene glycol may be varied, other biocompatible polymers may replace polyethylene glycol, e.g.,polyvinyl pyrrolidone, and other sugars or polysaccharides may substitute for dextrose.

[00126] Alternatively, other delivery systems for hydrophobic molecules may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Liposomal delivery systems are discussed above in the context of gene-delivery systems. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using sustained-release systems, such as semi-permeable matrices of solid hydrophobic polymers containing the effective amount of the composition to be administered. Various sustained-release materials are established and available to those of skill in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days. Depending on the chemical nature and

the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

[00127] For any composition used in the present methods of treatment, a therapeutically effective dose can be estimated initially using a variety of techniques well known in the art. For example, based on information obtained from a cell culture assay, a dose can be formulated in animal models to achieve a circulating concentration range that includes the $IC_{50}$. Similarly, dosage ranges appropriate for human subjects can be determined, for example, using data obtained from cell culture assays and other animal studies.

[00128] A therapeutically effective dose of an agent refers to that amount of the agent that results in amelioration of symptoms or a prolongation of survival in a subject. Toxicity and therapeutic efficacy of such molecules can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., by determining the $LD_{50}$ (the dose lethal to 50% of the population) and the $ED_{50}$ (the dose therapeutically effective in 50% of the population). The dose ratio of toxic to therapeutic effects is the therapeutic index, which can be expressed as the ratio $LD_{50}/ ED_{50}$. Agents that exhibit high therapeutic indices are preferred.

[00129] Dosages preferably fall within a range of circulating concentrations that includes the $ED_{50}$ with little or no toxicity. Dosages may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration, and dosage should be chosen, according to methods known in the art, in view of the specifics of a subject's condition.

[00130] Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety that are sufficient to modulate HIF$\alpha$ stabilization and HIF-regulated gene induction, as desired, i.e., minimal effective concentration (MEC). The MEC will vary for each compound but can be estimated from, for example, *in vitro* data. Dosages necessary to achieve the MEC will depend on individual characteristics of the compound and the route of administration. Agents or compositions thereof should be administered using a regimen which maintains plasma levels above the MEC for about 10-90% of the duration of treatment, preferably about 30-90% of the duration of treatment, and most preferably between 50-90%. In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

[00131] The amount of agent or composition administered will, of course, be dependent on a variety of factors, including the sex, age, and weight of the subject being treated, the severity of the affliction, the manner of administration, and the judgment of the prescribing physician.

[00132] The present compositions may, if desired, be presented in a pack or dispenser device containing one or more unit dosage forms containing the active ingredient. Such a pack or device may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition. Suitable conditions indicated on the label may include treatment of disorders or diseases in which ischemia or hypoxia is a major indication.

Compound Screening and Identification

[00133] The present invention further provides methods of screening for and identifying additional compounds that inhibit HIFα hydroxylation, or that stabilize HIFα, etc.

[00134] Various assays and screening techniques, including those described below, can be used to identify small molecules that modulate (e.g., increase or decrease) the level or activity of HIFα. Assays will typically provide for detectable signals associated with the consumption of a reaction substrate or production of a reaction product. Detection can involve, for example, fluorophores, radioactive isotopes, enzyme conjugates, and other detectable labels well known in the art. The results may be qualitative or quantitative. Isolation of the reaction product may be facilitated by a label, such as biotin or a histidine tag that allows purification from other reaction components via precipitation or affinity chromatography.

[00135] Assays for HIFα hydroxylation may involve measuring hydroxylated proline or lysine residues in HIFα or a fragment thereof (see, e.g., Palmerini et al. (1985) J Chromatogr 339:285-292), or measuring formation of succinate from 2-oxoglutarate in the presence of enzyme and HIFα or a fragment thereof (see, e.g., Cunliffe et al. (1986) Biochem J 240:617-619). Exemplary procedures that measure HIFα hydroxylation are described in Ivan et al. (*supra*) and Example 10. An exemplary procedure that measures production of succinate from 2-oxoglutarate is described by Kaule and Gunzler. (1990; Anal Biochem 184:291-297.) Substrate molecules may include HIFα or a fragment thereof, e.g., HIF(556-575); for example, an exemplary substrate for use in the assay described in

Example 10 is [methoxycoumarin]-DLDLEALAPYIPADDDFQL-amide (SEQ ID NO:5). Enzyme may include, e.g., HIFα prolyl hydroxylase (see, e.g., GenBank Accession No. AAG33965, etc.), obtained from any source. Enzyme may also be present in a crude cell lysate or in a partially purified form. Compounds that stabilize HIFα or that inhibit hydroxylation of HIFα may be identified by measuring and comparing enzyme activity in the absence and presence of the compound.

[00136] Additionally and in combination with the above methods, compounds can be identified by any of a variety of screening techniques known in the art. Such screening methods may allow for target polypeptides or the compounds to be free in solution, affixed to a solid support, borne on a cell surface, or located within a cell. For example, test compounds may be arrayed on a surface and analyzed for activity in a manner analogous to array methods currently available in the art. (See, e.g., Shalon et al. (1995) International Publication No. WO 95/35505; Baldeschweiler et al. (1995) International Publication No. WO 95/251116; Brennan et al. (1995) U.S. Patent No. 5,474,796; and Heller et al. (1997) U.S. Patent No. 5,605,662.)

[00137] These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein, and are specifically contemplated.

**EXAMPLES**

[00138] The invention is understood by reference to the following examples, which are intended to be purely exemplary of the invention. The present invention is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only. Any methods that are functionally equivalent are within the scope of the invention. Various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications fall within the scope of the appended claims.

**Example 1: HIFα Stabilization in Cells *in vitro***

[00139] Human cells derived from adenovirus-transformed fetal kidney epithelium (293A), cervical epithelial adenocarcinoma (HeLa), hepatocellular carcinoma (Hep3B), foreskin fibroblast (HFF), mammary gland epithelial adenocarcinoma (MCF7), umbilical vein endothelium (HUVEC), microvascular endothelium (HMEC-1), squamous carcinoma (SSC-25), lung fibroblast (HLF), and venous endothelium (AG10774B) tissues (see, e.g., American Type Culture Collection, Manassas VA; and Qbiogene, Carlsbad CA) were separately seeded

into 35 mm culture dishes and grown at 37°C, 20% $O_2$, 5% $CO_2$ in media as follows: HeLa cells in Dulbecco's Modification of Eagle's Medium (DMEM), 2% fetal bovine serum (FBS); HFF and HLF cells in DMEM, 10%FBS; 293A cells in DMEM, 5%FBS; HUVEC and AG10774B cells in Endothelial Growth Media (EGM-2; BioWhittaker, Inc., Walkersville MD); and HMEC-1 in RPMI 1640, 10%FBS; and Hep3B cells in Minimal Essential Medium (MEM), Earle's BSS (Mediatech Inc., Herndon VA), 2mM L-glutamine, 0.1mM non-essential amino acids, 1 mM sodium pyruvate, 10% FBS. When cell layers reached confluence, the media was replaced with OPTI-MEM media (Invitrogen Life Technologies, Carlsbad CA) and cell layers were incubated for approximately 24 hours in 20% $O_2$, 5% $CO_2$ at 37°C. Compound of the invention (one of compounds A to O) or DMSO (0.5 to 1%) was then added to existing medium, and incubation was continued overnight.

**[00140]** Following incubation, the media was removed, centrifuged, and stored for analysis (see below). The cells were washed two times in cold phosphate buffered saline (PBS) and then lysed in 1 ml of 10 mM Tris (pH 7.4), 1 mM EDTA, 150 mM NaCl, 0.5% IGEPAL (Sigma-Aldrich, St. Louis MO), and a protease inhibitor mix (Roche Molecular Biochemicals) for 15 minutes on ice. Cell lysates were centrifuged at 3,000xg for 5 minutes at 4°C, and the cytosolic fractions (supernatant) were collected. The nuclei (pellet) were resuspended and lysed in 100 µl of 20 mM HEPES (pH 7.2), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and a protease mix (Roche Molecular Biochemicals), centrifuged at 13,000xg for 5 minutes at 4°C, and the nuclear protein fractions (supernatant) were collected.

**[00141]** Nuclear fractions were normalized based on protein concentration and loaded onto a 4-12% TG gel and fractionated under reducing conditions. Proteins were transferred to a PVDF membrane (Invitrogen Corp., Carlsbad CA) at 500 mA for 1.5 hours. The membrane was blocked in T-TBS, 2% milk for 1 hour at room temperature and incubated overnight with mouse anti-human HIF-1α antibody (BD Biosciences, Bedford MA), diluted 1:250 in T-TBS, 2% milk. The blot was developed using SUPERSIGNAL WEST chemiluminescent substrate (Pierce, Rockford IL). As can be seen in Figures 1A, various compounds of the invention (Compounds A to F) stabilized HIFα in a normoxic environment in a dose-dependent manner, allowing HIFα to accumulate within the cell. As seen in Figure 1B, various cell types, including fibroblasts, epithelial cells, endothelial cells, and hepatocytes from various sources, showed dose-dependent stabilization of HIFα when treated with a compound of the invention in a normoxic environment.

**[00142]** Alternatively, nuclear and cytosolic fractions as prepared above were analyzed for HIF-1α using a QUANTIKINE immunoassay (R&D Systems, Inc., Minneapolis

MN) according to the manufacturer's instructions. As shown in Figure 2A, epithelial cells (293A) and hepatocytes (Hep3B) treated with various compounds of the invention (Compounds B and G to O) showed stabilization and accumulation of HIF$\alpha$ as compared to vehicle-treated control cells. As shown in Figure 2B, cells treated with compounds of the invention showed dose-dependent stabilization of HIF$\alpha$.

**Example 2: Effect on Oxygen Consumption**

**[00143]** Oxygen Sensor cell culture plates (BD Biosciences, Bedford MA) contain a ruthenium complex which is more fluorescent in the absence of oxygen. Therefore, the fluorescent read-out is increased by the presence of oxygen-consuming cells in the plate, which change the equilibrium to lower oxygen saturation and higher fluorescence. A compound that stabilizes HIF by inhibiting hydroxylation is expected to decrease oxygen consumption by decreasing oxygen consumed by the hydroxylation event itself and/or by shifting cellular metabolism from aerobic to anaerobic energy production.

**[00144]** Human cells derived from adenovirus-transformed fetal kidney epithelium (293A) or cervical epithelial adenocarcinoma (HeLa) (American Type Culture Collection) were grown to confluence in media (high glucose DMEM (Mediatech, Inc., Herndon VA), 1% penicillin/streptomycin mixture (Mediatech), 1% fetal bovine serum) at 37°C, 10% $CO_2$. Cells were collected and resuspended in media at a density of 500,000 cells/ml. The cell suspension was distributed at 0.2 ml/well into each well of an Oxygen Biosensor 96-well cell culture plate (BD Biosciences). The following treatments were added in 10 $\mu$l volumes to triplicate sets of wells: (1) 0.5% DMSO; (2) 200 $\mu$M sodium dodecyl sulfate; or (3) 1, 10, or 50 $\mu$M compound (one of compounds B, G, or a prodrug of compound V [pV]).

**[00145]** Cultures were incubated at 37°C, 10% $CO_2$ for 72 hours and plates were then read in an FL600 flourimeter (Biotek Instruments, Inc., Winooski VT) at an excitation wavelength of 485 nm and emission wavelength of 590 nm. Data was plotted as a function of fold change relative to DMSO control ($O_2$ consumption) or absorbance at a wavelength of 450 nm (WST-1) and descriptive statistical analysis was performed using EXCEL software (Microsoft Corporation, Bellevue WA).

**[00146]** Figure 3A shows the fold change in oxygen consumtion in cells treated with compound relative to control cells. As can be seen in the figure, all of the compounds produced a decrease in oxygen consumtion to some degree. Further, the reduction in oxygen consumption was dose-dependent (Figure 3A), and even at the highest doses little to no loss of cell viability was detected (Figure 1B). Additional experiments (not shown) in various cell

culture test systems, including incorporation of $^{3}$H-thymidine and total incorporation of amino acids, confirmed that the decrease in oxygen consumption was not associated with cytotoxicity.

**Example 3: Expression of HIF-Regulated Genes *in vitro***

[00147] Conditioned media collected from cell cultures grown as in Example 1 was analyzed for vascular endothelial growth factor (VEGF) expression using a QUANTIKINE immunoassay (R&D Systems) according to the manufacturer's instructions. As seen in Figure 4A, fibroblasts (HFF), epithelial cells (293A), and hepatocytes (Hep3B) treated with various compounds of the invention (one of compounds A, B, C, H, K, L, Q, and a prodrug of compound V [pV]) showed an increase in VEGF expression (Figure 4A). Values on the y-axis represent fold-induction relative to control and are reported on a $\log_2$ scale, such that a value of 1 represents 2-fold induction.

[00148] Alternatively, human cells derived from adenovirus-transformed fetal kidney epithelium (293A) were cultured in DMEM, 5% FBS, 1% Penicillin-Streptomycin at 37°C and 10% $CO_2$. After 48 hours, the cells were harvested and were plated confluent in 35 mm culture dishes in regular culture media, and after 1 day the media was changed to Opti-Mem I. After 18 to 24 hours, compound B was added to the media and incubation was continued for an additional 18 hours. Culture supernatant was then removed, the plates were placed on ice, lysis buffer (LB)-1 was added and the cells were harvested by scraping. The scraped cells were collected and incubated for 15 minutes on ice followed by centrifugation at 3000g for 5 minutes at 4°C. The supernatant, which represents the cytosolic fraction, was collected and cytosolic proteins were separated under denaturing and reducing conditions using SDS polyacrylamide gels that were loaded with equal amounts of protein per lane.

[00149] Gel electrophoresis was conducted at 150 V for 2 hours, and after SDS-PAGE the proteins were transferred to a PVDF membrane for 1.5 hours at 400 mA at 4°C. The membrane was then incubated in blocking buffer, washed once with T-TBS, and then anti-aldolase antibody diluted to working concentration in blocking buffer was added and the blots were incubated over night with gentle agitation at 4°C. The membrane was then washed 4 times with T-TBS, followed by incubation for one hour at room temperature with blocking buffer containing labeled secondary antibody. The membrane was then washed four times with T-TBS. The antigen specific for the primary antibody was visualized by exposing X-ray-film and developed using the ECL SUPERSIGNAL WEST FEMTO or PICO chemiluminescent substrate (Pierce, Rockford IL) according to the manufacturer's instructions.

[00150] Figure 4B shows that the compound increased expression of aldolase, an enzyme involved in glycolysis, over time. Thus, stabilization of HIFα by compounds of the invention leads to subsequent increase in expression of HIF-regulated genes.

**Example 4: HIFα Stabilization in Cells *in vivo***

[00151] Swiss Webster male mice (30-32 g) are obtained, e.g., from Charles River Laboratories, Inc. (Wilmington MA), or Simonsen, Inc. (Gilroy, CA), and treated by oral gavage one or more times per day for at least one day with a 2 ml/kg volume of either 0.5% carboxymethyl cellulose (CMC; Sigma-Aldrich) (control) or 5.0% compound (0.5% CMC). At one or more time points after the final dose, e.g., two and five hours, animals are anesthetized with isoflurane and 0.1 ml blood is collected, e.g., from the orbital sinus into a heparinized tube. After all selected time points have been reached, animals are subjected to a sub-lethal dose of $CO_2$ and blood is collected from the abdominal vein into a heparinized tube. All blood samples are stored at -80°C.

[00152] Tissues isolated from animals treated with compounds of the invention as described above are analyzed for HIFα protein levels as follows. Tissues are homogenized in 3 ml of 10 mM Tris (pH 7.4), 1 mM EDTA, 150 mM NaCl, 0.5% IGEPAL (Sigma-Aldrich), and a protease inhibitor mix (Roche Molecular Biochemicals) for 15 seconds using a POLYTRON PT-1200 homogenizer (Brinkmann Instruments, Inc., Westbury NY). Cell lysates are centrifuged at 3,000xg for 5 minutes at 4°C, and the cytosolic fraction (supernatant) is collected. The nuclei (pellet) are resuspended and lysed in 100 μl of 20 mM HEPES (pH 7.2), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and a protease mix (Roche Molecular Biochemicals), centrifuged at 13,000xg for 5 minutes at 4°C, and the nuclear protein fraction (supernatant) is collected.

[00153] Nuclear fractions are normalized based on protein concentration and loaded onto a 4 to 12% TG gel and fractionated under reducing conditions. Proteins are transferred to a PVDF membrane (Invitrogen Life Technologies) at 500 mA for 1.5 hours. The membrane is blocked in T-TBS, 2% milk for 1 hour at room temperature and incubated overnight with anti-HIFα antibody diluted in T-TBS, 2% milk. The blot is developed using SUPERSIGNAL WEST PICO chemiluminescent substrate (Pierce, Rockford IL).

[00154] Alternatively, nuclear and cytosolic fractions as prepared above are analyzed for HIF-1α using a QUANTIKINE immunoassay (R&D Systems) according to the manufacturer's instructions.

**Example 5: Expression of HIF-Regulated Genes *in vivo*.**

Experiment I

[00155] Twenty four Swiss Webster male mice (30-32 g) were obtained from Simonsen, Inc., and treated by oral gavage with a 4 ml/kg volume of either 0.5% CMC (Sigma-Aldrich) (0 mg/kg/day) or 1.25% Compound A (25 mg/ml in 0.5% CMC) (100 mg/kg). At 4, 8, 16, 24, 48, or 72 hours after the final dose, animals were anesthetized with isoflurane and a blood sample was collected from the abdominal vein. The blood sample was collected into a MICROTAINER serum separator tube (Becton-Dickinson, Franklin Lakes NJ), incubated at room temperature for 30 minutes, centrifuged at 8,000 rpm at 4°C for 10 min, and cell pellet was resuspended in RNALATER solution (Ambion) and stored at -80°C. The mice were then sacrificed and tissue samples of kidney, liver, brain, lung, and heart were isolated and stored in RNALATER solution (Ambion) at -80°C.

[00156] RNA isolation was carried out using the following protocol. A 50 mg section of each organ was diced, 875 μl of RLT buffer (RNEASY kit; Qiagen Inc., Valencia CA) was added, and the pieces were homogenized for about 20 seconds using a rotor-stator POLYTRON homogenizer (Kinematica, Inc., Cincinnati OH). The homogenate was micro-centrifuged for 3 minutes to pellet insoluble material, the supernatant was transferred to a new tube and RNA was isolated using an RNEASY kit (Qiagen) according to the manufacturer's instructions. The RNA was eluted into 80μL of water and quantitated with RIBOGREEN reagent (Molecular Probes, Eugene OR). Genomic DNA was then removed from the RNA using a DNA-FREE kit (Ambion Inc., Austin TX) according to the manufacturer's instructions. The absorbance at 260 and 280 nm was measured to determine RNA purity and concentration.

[00157] Alternatively, tissue samples were diced and homogenized in TRIZOL reagent (Invitrogen Life Technologies, Carlsbad CA) using a rotor-stator POLYTRON homogenizer (Kinematica). Homogenates were brought to room temperature, 0.2 volumes chloroform was added, and samples were mixed vigorously. Mixtures were incubated at room termperature for several minutes and then were centrifuged at 12,000g for 15 min at 4°C. The aqueous phase was collected and 0.5 volumes of isopropanol were added. Samples were mixed, incubated at room temperature for 10 minutes, and centrifuged for 10 min at 12,000g at 4°C. The supernatant was removed and the pellet was washed with 75% EtOH and centrifuged at 7,500g for 5 min at 4°C. Genomic DNA was then removed from the RNA using a DNA-FREE kit (Ambion Inc., Austin TX) according to the manufacturer's

instructions. The absorbance at 260 and 280 nm was measured to determine RNA purity and concentration.

[00158] RNA was precipitated in 0.3 M sodium acetate (pH 5.2), 50 ng/ml glycogen, and 2.5 volumes of ethanol for one hour at -20°C. Samples were centrifuged and pellets were washed with cold 80% ethanol, dried, and resuspend in water. Double stranded cDNA was synthesized using a T7-(dT)24 first strand primer (Affymetrix, Inc., Santa Clara CA) and the SUPERSCRIPT CHOICE system (Invitrogen) according to the manufacturer's instructions. The final cDNA was extracted with an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol using a PHASE LOCK GEL insert (Brinkman, Inc., Westbury NY). The aqueous phase was collected and cDNA was precipitated using 0.5 volumes of 7.5 M ammonium acetate and 2.5 volumes of ethanol. Alternatively, cDNA was purified using the GENECHIP sample cleanup module (Affymetrix) according to the manufacturer's instructions.

[00159] Biotin-labeled cRNA was synthesized from the cDNA in an *in vitro* translation (IVT) reaction using a BIOARRAY HighYield RNA transcript labeling kit (Enzo Diagnostics, Inc., Farmingdale NY) according to the manufacturer's instructions. Final labeled product was purified and fragmented using the GENECHIP sample cleanup module (Affymetrix) according to the manufacturer's instructions.

[00160] Hybridization cocktail was prepared by bringing 5 μg probe to 100 μl in 1x hybridization buffer (100 mM MES, 1 M [$Na^+$], 20 mM EDTA, 0.01% Tween 20), 100 μg/ml herring sperm DNA, 500 μg/ml acetylated BSA, 0.03 nM contol oligo B2 (Affymetrix), and 1x GENECHIP eukaryotic hybridization control (Affymetrix). The cocktail was sequentially incubated at 99°C for 5 minutes and 45°C for 5 minutes, and then centrifuged for 5 minutes. The Murine genome U74AV2 array (MG-U74Av2; Affymetrix) was brought to room temperature and then prehybridized with 1x hybridization buffer at 45°C for 10 minutes with rotation. The buffer was then replaced with 80 μl hybridization cocktail and the array was hybridized for 16 hours at 45°C at 60 rpm with counter balance. Following hybridization, arrays were washed once with 6x SSPE, 0.1% Tween 20, and then washed and stained using R-phycoerythrin-conjugated streptavidin (Molecular Probes, Eugene OR), goat anti-streptavidin antibody (Vector Laboratories, Burlingame CA), and a GENECHIP Fluidics Station 400 instrument (Affymetrix) according to the manufacturer's micro_1v1 protocol (Affymetrix). Arrays were analyzed using a GENEARRAY scanner (Affymetrix) and Microarray Suite software (Affymetrix).

[00161] The Murine Genome U74AV2 array (Affymetrix) represents all sequences (~6,000) in Mouse UniGene database build 74 (National Center for Biotechnology Information, Bethesda MD) that have been functionally characterized and approximately 6,000 unannotated expressed sequence tag (EST) clusters.

[00162] As seen in Figure 5A, expression of genes encoding angiogenic proteins was increased in a coordinated fashion after treatment with a compound of the invention in lung, a representative organ. Transcript patterns represented in the figure include VEGF-C, Flt-1/VEGF receptor-1, adrenomedullin, endothelin-1, plasminogen activator inhibitor (PAI)-1, and Cyr61. In the time course, mRNA levels peak early, then return to control levels after 24 hours. Figure 5B shows the specific expression time course for two genes, endothelin-1 and adrenomedullin, representative of the cluster of genes shown in Figure 5A. In similar experiments, a significant increase was also seen for additional HIF-regulated genes including, e.g., phosphofructokinase, enolase 1, lactate dehydrogenase, glucose transporter 1, acyl CoA thioesterase, heme oxygenase, transferrin receptor, IGFBP-1, nip3, nix, and cyclin G3.

[00163] As can be seen in Figure 7A, expression of genes encoding glycolytic enzymes was increased in a coordinated fashion after treatment with a compound of the invention in kidney, a representative organ. Transcript patterns represented in the figure include aldolase-A, enolase-1, glucose transporters (GluT)-1 and -3, GAPDH, hexokinase-1 and -2, lactate dehydrogenase-A, phosphofructokinase-L and -C, phosphoglycerate kinase-1, and pyruvate kinase-M. In the time course, mRNA levels peak early, then return to control levels after 24 hours. Figure 7B shows the specific expression time course for two genes, aldolase and phosphofructokinase, representative of the cluster of genes shown in Figure 7A.

Experiment II

[00164] Twelve Swiss Webster male mice (30-32 g) were obtained from Simonsen, Inc., and treated by oral gavage two times per day for 2.5 days (5 doses) with a 4 ml/kg volume of either 0.5% CMC (Sigma-Aldrich) (0 mg/kg/day) or 2.5% compound (B or E; 25 mg/ml in 0.5% CMC) (200 mg/kg/day). Four hours after the final dose, animals were anesthetized with isoflurane and a blood sample was collected from the abdominal vein. The blood sample was collected into a MICROTAINER serum separator tube (Becton-Dickinson), incubated at room temperature for 30 minutes, centrifuged at 8,000 rpm at 4°C for 10 min, and then the serum fraction was processed and analyzed for vascular endothelial growth factor (VEGF) expression using a QUANTIKINE immunoassay (R&D Systems) according to

the manufacturer's instructions. The mice were then sacrificed and approximately 150 mg of liver and each kidney were isolated and stored in RNALATER solution (Ambion) at -20°C.

[00165] RNA isolation was carried out using the following protocol. Tissue slices were cut into small pieces, 1.75 ml of RLT lysis buffer (RNEASY kit; Qiagen) was added, and the pieces were homogenized for about 20 seconds using a rotor-stator POLYTRON homogenizer (Kinematica, Inc., Cincinnati OH). A 350 μl volume of homogenate was micro-centrifuged for 3 minutes to pellet insoluble material, the supernatant was transferred to a new tube and RNA was isolated using an RNEASY kit (Qiagen) according to the manufacturer's instructions. The RNA was eluted into 80μL of water and quantitated with RIBOGREEN reagent (Molecular Probes, Eugene OR). Genomic DNA was then removed from the RNA using a DNA-FREE kit (Ambion) according to the manufacturer's instructions. The absorbance at 260 and 280 nm was measured to determine RNA purity and concentration.

[00166] cDNA synthesis was performed using 1μM random hexamer primers, 1μg of total RNA, and OMNISCRIPT reverse transcriptase (Qiagen), according to the manufacturer's instructions. Resulting cDNA was diluted 5-fold with water to give 100μL final volume. Analysis of the relative level of vascular endothelial growth factor (VEGF) gene expression was performed by quantitative PCR using a FASTSTART DNA MASTER SYBR GREEN I kit (Roche Molecular Biochemicals) and VEGF-specific primers, using a LIGHTCYCLER system (Roche Molecular Biochemicals), according to manufacturer's instructions. Samples were heated to 94°C for 6 minutes and then cycled through 95°C for 15 seconds, 60°C for 5 seconds, and 72°C for 10 seconds for a total of 42 cycles. VEGF-specific primers were as follows:

| | | |
|---|---|---|
| m-VEGF-F1 | GTTGCAAGGCGAGGCAGCTT | (SEQ ID NO:1) |
| m-VEGF-R1 | TGACGATGATGGCATGGTGGT | (SEQ ID NO:2) |

[00167] The relative level of 18S ribosomal RNA gene expression was measured as a control. Quantitative PCR was performed using a QUANTITECT SYBR GREEN PCR kit (Qiagen) and 18S rRNA-specific primers, using a LIGHTCYCLER system (Roche Molecular Biochemicals), according to manufacturer's instructions. Samples were heated to 95°C for 15 minutes and then cycled through 94°C for 15 seconds, 60°C for 20 seconds, 72°C for 10 seconds for a total of 42 cycles. Ribosomal RNA-specific primers were as follows:

| | | |
|---|---|---|
| 18S-rat-2B | TAGGCACGGCGACTACCATCGA | (SEQ ID NO:3) |
| 18S-rat-2A | CGGCGGCTTTGGTGACTCTAGAT | (SEQ ID NO:4) |

[00168] Each PCR run included a standard curve and water blank. In addition, a melt curve was run after completion of each PCR run to assess the specificity of the amplification. VEGF gene expression was normalized relative to the expression level of 18S ribosomal RNA for that sample.

[00169] Figure 6A shows compound E increased VEGF expression in kidney and compound B increased VEGF expression in liver and kidney. As can be seen in Figure 6B, levels of VEGF in the plasma of animals treated with compound are significantly increased relative to untreated control animals at 2, 5, and 20 hours after the final dose.

**Example 6: Cardiac Ischemia**

Experiment I

[00170] Nwogu et al. (2001; Circulation 104:2216-2221) reported the use of a compound of the invention following myocardial infarction. Although the authors interpreted their results relative to the compounds affect on fibrosis, the present invention clearly shows that the primary benefit on heart performance is due to stabilization of HIFα. Experiments are as described in Nwogu et al. (*supra*) and as described below.

[00171] Seventy adult male Wistar rats (200-250 g) were anaesthetized and subjected to left coronary artery occlusion to produce acute myocardial infarction (AMI). Nine animals were subjected to identical surgery without coronary artery ligation. Twenty-four to forty-eight hours after surgery, electrocardiogram (ECG) electrodes were applied to the paws and a 15 MHz linear probe (Acuson Corp., Mountain View CA) was applied to the chest to obtain short axis transthoracic echocardiography (2DE) images at the mid-papillary muscle level. The probe was moved cephalad or caudad and angulated until clear endocardial visualization of the left ventricular cavity was detected. Images were obtained using the Sequoia ultrasound system (Acuson). Animals with less than 20% fractional shortening and regional wall motion abnormality on the 2DE were randomized to treatment with compound A (n=14) or vehicle (n=12). The sham controls were also randomized to treatment with compound A (n=4) or vehicle (n=5).

[00172] Animals were treated by gavage 2 times per day for the duration of the experiment with compound A at 50 mg/kg or with vehicle alone. Serum level of drug was determined periodically to establish that treated animals received sufficient and consistent amount of drug and that the measured levels were sufficient to inhibit prolyl 4-hydroxylase, a representative 2-oxoglutarate dioxygenase.

[00173] Serial 2DE images were obtained weekly. Three short axis 2DE digital clips containing 5 or more systolic and diastolic frames were captured and stored. Two observers blinded to treatment did measurements off-line. For the measurements, the digital images were slowed and frozen at end systole and end diastole. Two systolic and two diastolic frames from each of the three digital clips were measured by consensus and averaged. The anterior wall in systole (AWS) and diastole (AWD), posterior wall in systole (PWS) and diastole (PWD), and left ventricular end systolic (LVESD) and end diastolic (LVEDD) were measured according to the American Society for Echocardiology (ASE) leading-edge method. For consistency, measurements were done from the anterior to the posterior mid points of the left ventricular cavity and were randomly repeated to ensure reproducibility (reproducibility was approximately 96%).

[00174] At four weeks of treatment, *in vivo* hemodynamic measurements were determined, as described below, both before and after infusion (0.2 ml over 1 minute) of $10^{-7}$ M isoproterenol via the femoral vein. Hearts were then excised and weighed as described below.

[00175] Alternatively, one hundred forty adult male Wistar rats (200-250 g) were anaesthetized and subjected to left coronary artery occlusion to produce AMI. Forty-eight hours after surgery, 2DE images were obtained and animals with a significant area of infarction were randomized to treatment with compound A (n=34) or vehicle (n=34).

[00176] Animals were treated by gavage 2 times per day for the duration of the experiment with compound A at 50 mg/kg or with vehicle alone. Serum level of drug was determined periodically to establish that treated animals received sufficient and consistent amount of drug and that the measured levels were sufficient to inhibit prolyl 4-hydroxylase.

[00177] Digital mid-papillary muscle and apical four chamber 2DE images were obtained biweekly on half of the animals in each group until week 8. Two observers blinded to treatment did measurements off-line. For the measurements, the digital images were slowed and frozen at end systole and end diastole. Two to three endocardial surfaces were traced in both the short axis and four chamber views and averaged. The left venticular area in systole and diastole, ejection fraction, fractional area change, wall thickness, mitral peak E wave velocity, aortic peak velocity, and infarct size were measured.

[00178] After 10 weeks of treatment, *in vivo* hemodynamic measurements were determined and hearts were excised and weighed as described below.

[00179] To collect *in vivo* hemodynamic measurements, animals were anaesthetized and the right carotid artery was dissected free from surrounding tissues and canulated with an SPR-671 ultra-miniature pressure transducer (Millar Instruments, Inc., Houston TX). The catheter was then advanced into the left ventricle. After steady state was established, baseline heart rate (HR), developed pressure (DP), contractile index (CI), left ventricle systolic pressure (SBP) and end diastolic pressure (LVEDP), and maximal rate of pressure rise and fall (+dP/dt and -dP/dt, respectively) were recorded.

[00180] Following hemodynamic measurement, hearts were excised and weighed. Pieces of scarred myocardium, and right ventricle and left ventricle myocardium distant from the site of infarct were dissected out and weighed. Hydroxyproline and proline were determined by the method of Palmerini et al. (1985, J Chromatogr 339:285-92) except that L-azetidine-2-carboxylic acid (Sigma-Aldrich) was substituted for 3,4-dehydroproline as the internal standard.

[00181] An immediate reduction in mortality was seen when compounds of the invention were administered following myocardial infarction. As can be seen in Figure 8, no deaths were seen in the treated group immediately following insult to the heart, and over 90% of the treated group was still alive 8 weeks later. In comparison, only about 60% of the untreated group survived this period. Statistically significant improvement in survival ($P<0.05$) in the treated group relative to untreated group was seen at weeks 2 through 8, with a relative reduction in mortality of 77%.

[00182] Heart parameters were also improved in the treated group over the untreated group. Table 1 shows no increase in left ventricle end diastolic diameter (LVEDD) in the treated group, whereas the untreated group shows an increase in both LVEDD and left ventricle end systolic diameter (LVESD) measures over the same time period. The dilation of the heart in the untreated group was statistically different in the treated group relative to the untreated group after 1 week of treatment.

**Table 1.** Changes in left ventricle end diastolic diameter.

| Week | Treated-MI (mm) | Untreated-MI (mm) | Sham (mm) |
|---|---|---|---|
| 0 | 69 ± 1 | 67 ± 2 | 43 ± 3 |
| 1 | 68 ± 2 | 76 ± 2 | 44 ± 3 |
| 2 | 69 ± 3 | 74 ± 4 | 45 ± 2 |
| 3 | 68 ± 4 | 75 ± 3 | 45 ± 2 |

Values in the table represent the mean ± standard deviation.

**Table 2.** Changes in left ventricle end systolic diameter.

| Week | Treated-MI (mm) | Untreated-MI (mm) | Sham (mm) |
|---|---|---|---|
| 0 | 77 ± 2 | 75 ± 1 | 67 ± 2 |
| 1 | 82 ± 2 | 88 ± 1 | 65 ± 2 |
| 2 | 85 ± 2 | 86 ± 3 | 69 ± 2 |
| 3 | 85 ± 3 | 86 ± 2 | 68 ± 4 |

Values in the table represent the mean ± standard deviation.

[00183] Figures 9A and 9B show graphical representations of the increase in LVESD and LVEDD, respectively, over time. The left ventricle end diastolic and systolic diameters were similar in the three groups at the time of randomization. Figure 10A shows statistically significant improvement in left ventricular ejection fraction (LVEF) in treated animals relative to untreated controls in weeks 2 through 8. At randomization, the LVEF for both groups was 33%. The apparent increase in LVEF between week 4 and week 6 in the untreated control group reflects the high mortality in members of this group.

[00184] Fractional shortening of the myocardium during contraction was also improved in the treated group. Table 3 shows statistically significant improvement in fractional shortening in the treated group relative to the untreated group in weeks 1 to 4.

Table 3. Changes in fractional shortening.

| Weeks | Treated-MI (%) | Untreated-MI (%) | Sham (%) |
|---|---|---|---|
| 0 | 10 ± 0.8 | 12 ± 1 | 34 ± 3 |
| 1 | 17 ± 1 | 13 ± 1 | 33 ± 3 |
| 2 | 20 ± 2 | 15 ± 2 | 33 ± 2 |
| 3 | 21 ± 2 | 12 ± 1 | 35 ± 2 |
| 4 | 21 ± 3 | 16 ± 2 | 36 ± 1 |

Values in the table represent the mean ± standard deviation.

[00185] Further, as can be seen in Figure 10B, fractional shortening in the treated group increased from 10% at baseline to 20% at week 2, a 79% increase relative to baseline. Both the untreated group and sham controls remained unchanged over the 4 week period.

[00186] The ability of the heart to contract and relax following trauma induced by cardiac ischemia was also improved in the treated group. Table 4A shows statistically significant differences in negative change in pressure over time (-dP/dt), a measure of the hearts ability to relax following contraction, in the treated group relative to the untreated group following 4 weeks of treatment. As shown in Table 4A and in Figure 11, stimulation of the heart with isoproterenol shows statistically significant differences in positive change in pressure over time (+dP/dt), a measure of the hearts ability to contract, in the treated group relative to the untreated group.

Table 4A. Hemodynamic data at 4 weeks post-MI.

| | Treated-MI | Untreated-MI | Sham |
|---|---|---|---|
| **Systolic BP (mm Hg)** | | | |
| **baseline** | 143 ± 7 | 142 ± 3 | 144 ± 5 |
| **isoproterenol** | 130 ± 9 | 123 ± 7 | 197 ± 3 |
| **Developed pressure (mm Hg)** | | | |
| **baseline** | 133 ± 6 | 133 ± 3 | 135 ± 6 |
| **isoproterenol** | 121 ± 9 | 115 ± 8 | 173 ± 3 |
| **+dP/dt (mm Hg/sec)** | | | |
| **baseline** | 9477 ± 581 | 8642 ± 209 | 9925 ± 1194 |
| **isoproterenol** | 16830 ± 1195 | 13832 ± 1097 | 21515 ± 1074 |
| **-dP/dt (mm Hg/sec)** | | | |
| **baseline** | 9978 ± 827 | 8009 ± 426 | 11578 ± 622 |
| **isoproterenol** | 9234 ± 703 | 8984 ± 622 | 11549 ± 10742 |

Values in the table represent the mean ± standard deviation.

[00187] Table 4B shows statistically significant differences in both +dP/dt and -dP/dt in the treated group relative to the untreated group following 10 weeks of treatment.

**Table 4B.** Hemodynamic data at 10 weeks post-MI.

| | Treated-MI | Untreated-MI | P-value |
|---|---|---|---|
| **Systolic BP (mm Hg)** | 106 ± 4 | 92 ± 5 | 0.053 |
| **Developed pressure (mm Hg)** | 97 ± 3 | 69 ± 14 | 0.031 |
| **+dP/dt (mm Hg/sec)** | 6701 ± 331 | 4937 ± 828 | 0.042 |
| **-dP/dt (mm Hg/sec)** | 6395 ± 373 | 3641 ± 737 | 0.002 |

Values in the table represent the mean ± standard deviation.

**[00188]** Significant improvement was also seen at 10 weeks in developed pressure and systolic blood pressure in the treated group relative to the untreated group.

**[00189]** While it is recognized that recovery from an initial infarct requires connective tissue deposition within the necrotic region, the present invention demonstrates no adverse affects of treatment with respect to scar formation. On the contrary, as can be seen in Table 5A, there is no statistically significant change in collagen deposition in the scar and non-infarcted tissue at 4 weeks, demonstrating the improvement in heart performance in the first 4 weeks is unrelated to collagen deposition.

**Table 5A.** Collagen content in the heart at 4 weeks post-MI.

| | Treated-MI | Untreated-MI | Sham |
|---|---|---|---|
| **Hydroxyproline/proline in non-infarct left ventricular myocardium** | 0.12 ± 0.06 | 0.18 ± 0.05 | 0.11 ± 0.02 |
| **Hydroxyproline/proline in non-infarct right ventricular myocardium** | 0.13 ± 0.02 | 0.17 ± 0.03 | 0.15 ± 0.03 |
| **Hydroxyproline/proline in infarct scar** | 0.34 ± 0.08 | 0.45 ± 0.09 | - |

Values in the table represent the mean ± standard deviation.

**[00190]** However, as can be seen in Table 5B, there is a statistically significant absolute reduction in the collagen content of the non-infarcted myocardium and scar tissue of the treated group relative to the untreated group at 10 weeks, demonstrating that the methods of the present invention do reduce reactive cardiac fibrosis over a longer time course.

**Table 5B.** Collagen content in the heart at 10 weeks post-MI.

| | Treated-MI | Untreated-MI | P-value |
|---|---|---|---|
| **Hydroxyproline/proline in non-infarct left ventricular myocardium** | 0.099 ± 0.025 | 0.135 ± 0.036 | <0.05 |
| **Hydroxyproline/proline in non-infarct right ventricular myocardium** | 0.152 ± 0.044 | 0.175 ± 0.042 | - |
| **Hydroxyproline/proline in infarct scar** | 0.471 ± 0.024 | 0.638 ± 0.020 | <0.05 |

Values in the table represent the mean ± standard deviation.

Experiment II

**[00191]** Male Wistar rats (100-110 g), aged 4-5 weeks, were kept on a regular diet and a 12 hour day-night cycle. The animals were randomized into treatment regimens as follows: (1) Sham operated animals (n=12), (2) myocardial infarction controls (n=25), and (3) myocardial infarction with compound B treatment (n=25). Animals were treated for two days prior to surgery and for one week following surgery. Animals were treated by oral gavage two times per day with either 0.5% CMC (Sigma-Aldrich) (control) or 50 mg/kg compound B in 0.5% CMC. Ligation of the left anterior descending coronary artery was performed in artificially ventilated animals after left throacotomy. Animals were sacrificed one week after surgery and echocardiography was performed. Fractional shortening, end-diastolic diameters, and end-systolic diameters were determined in a blinded fashion.

**[00192]** As can be seen in Figure 12A, fractional shortening was reduced from 51% in sham-operated animals to 29% in untreated MI controls. Treatment with compound showed a statistically significant ($p<0.05$; one-way ANOVA/Turey's test) improvement in fractional shortening, to 41%, relative to the untreated control group. Similarly, Figure 12B shows statistically significant improvement in left ventricular end-diastolic (LVEDD) and end-systolic (LVESD) diameters in treated animals relative to untreated MI controls ($p<0.005$ and $p<0.001$, respectively; one-way ANOVA/Turey's test). Animals treated with compound showed no increase in left ventricular end-systolic diameter and an 18% increase in end-diastolic diameter over sham operated animals. The untreated controls, however, showed a 15% and 65% increase in LVESD and LVEDD, respectively.

**Example 7: Liver Ischemia**

**[00193]** Bickel et al. (1998; Hepatology 28:404-411) reported the use of a compound of the invention following induction of toxic-ischemic injury in the liver. Although the authors interpreted their results relative to the effect of the compounds on fibrosis, the authors acknowledged that the beneficial effects on variables of liver function including serum levels

of bilirubin, bile acids, and alkaline phosphatase could not be directly attributed to a reduction in fibrosis.

[00194] The model of toxic-ischemic liver injury was described in Bickel et al. (*supra*). Briefly, male Wistar rats (212-320 g) either received 1 ml/kg carbon tetrachloride ($CCl_4$) in olive oil (1:1) by gavage twice weekly for nine weeks (n=140) or received no treatment (controls; n=10). Additionally, a group of animals receiving $CCl_4$ (n=60) also received compound P. The compound was administered by intraperitoneal injection twice daily at 60 mg compound/2 ml saline/kg body weight. After 9 weeks, the animals were sacrificed and the liver was weighed. Bilirubin, alanine transaminase, alkaline phosphatase, albumin, and total bile acids in serum were determined using commercially available kits.

[00195] As can be seen in Table 6 (Bickel et al., *supra*, Table 2), induction of liver damage produced a significant reduction in body weight (BW), although no significant change in liver weight was seen (not shown).

**Table 6.** Serum parameters of liver function after 9 weeks of treatment.

| Treatment | N | BW (g) | BR (μmol/L) | tBA (μmol/L) | ALT (U/L) | AP (U/L) |
|---|---|---|---|---|---|---|
| Control | 10 | 425 ± 66.9 | 2.00 ± 0.50 | 8.48 ± 8.40 | 27.5 ± 10.9 | 156 ± 57.5 |
| $CCl_4$ | 80 | 370 ± 43.3 | 4.34 ± 3.93 | 81.3 ± 87.9 | 83.1 ± 51.7 | 269 ± 117 |
| $CCl_4$ + CPD | 60 | 373 ± 38.9 | 2.83 ± 2.21 | 40.8 ± 51.4 | 59.0 ± 29.5 | 195 ± 72.7 |

Values in the table represent the mean ± standard deviation.

[00196] Liver damage also produced a measurable and statistically significant decrease in liver function as determined by serum levels of bilirubin (BR), total bile acids (tBA), alanine transaminase (ALT), and alkaline phosphatase (AP), which increased 117%, 856%, 201%, and 72%, respectively. However, treatment with a compound of the invention (CPD) produced statistically significant improvement in liver function. Serum levels of BR, tBA, ALT, and AP decreased 64%, 65%, 43%, and 65%, respectively, in the treated group relative to the untreated group. The improvement in liver function is attributed to stabilization of HIF$\alpha$ by the methods of the invention.

**Example 8: Renal Ischemia-Reperfusion Injury**

[00197] The model of ischemic acute renal failure was described in Nemoto et al. (2001, Kidney Int 59:246-251.) Briefly, male Sprague-Dawley rats (200 - 250g) were treated with either 0.5% carboxymethyl cellulose (CMC; Sigma-Aldrich) or 1.5 % compound B

suspended in CMC by oral gavage in a volume of 4ml/kg/day. Rats were pretreated daily for 4 consecutive days (days -3 to 0). A few hours after the fourth and last oral dose on day 0, renal ischemia-reperfusion injury (IRI) was performed.

[00198] Animals were divided into four groups: (1) Vehicle pretreatment and sham surgery; (2) compound B pretreatment and sham surgery; (3) vehicle pretreatment and IRI surgery; and (4) compound B pretreatment and IRI surgery. Animals were anesthetized under isoflurane, an incision was made in the abdominal midline, and the renal pedicles were bluntly dissected. A vascular clip was placed on the right renal pedicle for 45 minutes while the left kidney underwent simultaneous nephrectomy. After each occlusion, the clip was released at 45 minutes, and reperfusion was observed by the changing color of the kidney. Temperature was maintained constant, and warm saline (0.5% of body weight) containing Buprenex analgesic was administered directly into abdomen before the incision was completely sutured.

[00199] The animal body weight and mortality were monitored. Blood samples were obtained from the tail vein, and serum chemistry and CBC were measured by IDEXX veterinary service (West Sacramento CA). Data are presented as mean ± SE with number of animals in parenthesis. The data were compared within the four groups at each time point using one-way analysis of variance (ANOVA, SIGMASTAT) and Student-Newman-Keuls method. A value of $P < 0.05$ was considered significant.

[00200] As can be seen in Figure 13, treatment with the compound prevented early mortality associated with ischemic-reperfusion injury. Further, serum blood urea nitrogen (BUN), a gauge of renal function, was significantly elevated by renal IRI at both 3 and 7 days, whereas treatment with compound produced significantly less IRI-induced increase in BUN. (Figure 14A.) Additionally, serum cholesterol was significantly elevated by renal IRI at days 3, 7 and 14, whereas treatment with compound completely blocked IRI-induced increase in serum cholesterol. (Figure 14B.) Athough the reasons are still under investigation, elevated kidney cholesterol is a natural reflection of renal ischemic-reperfusion injury. (Zager et al. (2001) Am J Pathol 159:743-752; Appel (1991) Kidney Int 39:169-183; and Abdel-Gayoum et al. (1999) Hum Exp Toxicol 18:454-459.)

**Example 9: Enhanced Granulation Tissue Formation in Chronic Wounds**

[00201] The ability to treat chronic wounds utilized the rabbit cutaneous hypertrophic scarring model described in Morris et al. (1997, Plast Reconstr Surg 100:674-681) and Marcus et al. (2000, Plast Reconstr Surg 105:1591-1599). Briefly, female New Zealand

White rabbits (n=12; 3-6 months of age) were anesthetized and four, 7-mm dermal ulcer wounds were created on the ventral surface of each ear with removal of the perichondrium. Wounds were treated and covered with TEGADERM semi-occlusive polyurethane dressing (3M Health Care, St. Paul MN). Wounds were treated by topical application of 0.5% or 1% (w/v) a prodrug of compound V [pV] in an aqueous 0.5% (w/v) CARBOPOL 971 PNF gel (pH 6.5; Noveon Inc., Cleveland OH) once per day for the first week. When tested *in vitro*, gels released 50% of the drug within 2 hrs and 95% of the drug within 4 hrs. The treatment ear received either a low-dose treatment (0.5% compound) or a high dose treatment (1% compound), while the control ear received gel alone. Treatment delivery was facilitated by creating a hole in the dressing applied at the time of wounding to prevent irritation of the area surrounding the wound by daily removal of dressing. The hole was then covered by a smaller piece of dressing to prevent wound desiccation. Wounds with obvious desiccation or infection were excluded from the study.

**[00202]** At post-wounding days 7 and 12, wounds were harvested, bisected, and stained with hemotoxylin-eosin for evaluation of granulation tissue formation and wound epithelialization. Observers blinded to treatment quantitated wound healing parameters in histological sections by the use of a graduated eyepiece reticle. Data were analyzed using the Student's t-test to compare treated and untreated samples. A $P<0.05$ was considered significant.

**[00203]** The wounds were evaluated for granulation tissue formation and wound epithelialization; parameters of wound healing that are sensitive ischemia and hypoxia. (Corral et al. (1999) Arch Surg 134:200-205; and Ahn and Mustoe (1990) Ann Plast Surg 24:17-23.) As shown in Figure 15A, an increase in granulation tissue area was seen in treated wounds relative to untreated wounds. As can be seen in Figure 15B, there was no difference in the peak-to-peak distance in treated versus untreated animals. The peak-to-peak value is an indicator of wound coverage by granulation tissue. Thus, the methods of the invention can be used to increase vascularization and granulation tissue formation in wounds, such as chronic wounds and ulcers.

**Example 10: Screening Assay**

**[00204]** Compounds that inhibit HIF-specific prolyl hydroxylase activity and thereby stabilize HIF$\alpha$ can be identified and characterized using the following assay. A 50 $\mu$l aliquot of a reaction mix containing 4 mg/ml BSA, 0.1 M Tris HCl (pH 7.2), 2 mM ascorbate, 80 $\mu$M ferrous sulfate, 0.2 mM 2-oxoglutarate, 600 units/ml catalase, with or without 100 $\mu$M HIF$\alpha$ peptide is mixed with 50 $\mu$l HeLa cell extract or purified HIF prolyl hydroxylase and

incubated 1.5 hours at 37°C. Following incubation, 50 μl of streptavidin beads are added and the mixture is incubated for 1 hour with agitation at 4°C. The mixture is transferred to tubes and centrifuged at low speed to pellet the beads. The beads are washed three times with 0.5 to 1 ml 20 mM Tris HCl (pH 7.2). The peptide is then eluted from the beads with 5 μl 2 mM biotin in 20 mM Tris HCl (pH 7.2) for 1 hour. The tubes are centrifuged to pellet the resin and 40-50 μl of supernatant is removed and an equal volume of acetonitrile is added. Alternatively, the peptide is attached to methoxycoumarin, a pH insensitive fluorophore. The fluorophore may provide sensitivity and specificity to enhance detection in assays run with crude cell lysate. An exemplary HIF peptide for use in the screening assay may comprise [methoxycoumarin]-DLDLEALAPYIPADDDFQL-amide (SEQ ID NO:5). The non-hydroxylated and hydroxylated peptides are then separated by reverse-phase HPLC on a C18 column with UV detection at 214 nm.

**[00205]** Various modifications of the invention, in addition to those shown and described herein, will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

**[00206]** All references cited herein are hereby incorporated by reference herein in their entirety.

What is claimed is:

1. A method for stabilizing the alpha subunit of hypoxia inducible factor (HIFα) in a subject, the method comprising administering a compound that inhibits hydroxylation of HIFα.

2. A method for stabilizing the alpha subunit of hypoxia inducible factor (HIFα) in a subject, the method comprising administering to a compound that inhibits 2-oxoglutarate dioxygenase enzyme activity.

3. A method for stabilizing the alpha subunit of hypoxia inducible factor (HIFα) in a subject, the method comprising administering to a subject a compound that inhibits HIF prolyl hydroxylase enzyme activity.

4. The method of claim 1, wherein HIFα is selected from the group consisting of HIF-1α, HIF-2α, HIF-3α, and any fragment thereof.

5. The method of claim 1, wherein the HIFα is endogenous to the subject.

6. The method of claim 2, wherein the 2-oxoglutarate dioxygenase enzyme is selected from the group consisting of EGLN1, EGLN2, EGLN3, procollagen prolyl 4-hydroxylase, procollagen prolyl 3-hydroxylase, procollagen lysyl hydroxylase, PHD4, FIH-1, and any subunit or fragment thereof.

7. The method of claim 3, wherein the HIF prolyl hydroxylase enzyme is selected from the group consisting of EGLN1, EGLN2, EGLN3, and any subunit or fragment thereof.

8. The method of claim 1, wherein the administering occurs *in vivo*.

9. The method of claim 8, wherein the subject is an animal.

10. The method of claim 8, wherein the subject is a mammal.

11. The method of claim 8, wherein the subject is a human.

12. The method of claim 1, wherein the administering occurs *ex vivo*.

13. The method of claim 12, wherein the subject is selected from the group consisting of a cell, a tissue, and an organ.

14. The method of claim 13, wherein the cell, tissue, or organ is derived from a system selected from the group consisting of the renal, cardiac, hepatic, pulmonary, hematopoietic, gastrointestinal, neuronal, and musculoskeletal systems.

15. A method for treating, preventing, or pretreating a HIF-associated condition in a subject, the method comprising stabilizing HIFα.

16. The method of claim 15, wherein the HIF-associated condition is associated with hypoxia.

17. The method of claim 15, wherein the HIF-associated condition is associated with ischemia.

18. A method for treating, preventing, or pretreating a HIF-associated condition in a subject, the method comprising inhibiting 2-oxoglutarate dioxygenase enzyme activity.

19. The method of claim 18, wherein the HIF-associated condition is associated with hypoxia.

20. The method of claim 18, wherein the HIF-associated condition is associated with ischemia.

21. A method for treating, preventing, or pretreating a HIF-associated condition in a subject, the method comprising inhibiting HIF prolyl hydroxylase enzyme activity.

22. The method of claim 21, wherein the HIF-associated condition is associated with hypoxia.

23. The method of claim 21, wherein the HIF-associated condition is associated with ischemia.

24. The method of claim 15, wherein the HIF-associated condition is a pulmonary disorder.

25. The method of claim 15, wherein the HIF-associated condition is a cardiac disorder.

26. The method of claim 15, wherein the HIF-associated condition is a neurological disorder.

27. The method of claim 15, wherein the HIF-associated condition is associated with an ischemic event.

28. The method of claim 27, wherein the ischemic event is acute.

29. The method of claim 28, wherein the ischemic event is associated with surgery, organ transplantation, infarction, trauma, or injury.

30. The method of claim 27, wherein the ischemic event is chronic.

31. The method of claim 30, wherein the ischemic event is associated with a condition selected from the group consisting of hypertension, diabetes, occlusive arterial disease, chronic venous insufficiency, Raynaud's disease, cirrhosis, congestive heart failure, and systemic sclerosis.

32. The method of claim 15, wherein the method comprises administering to the subject a compound that stabilizes HIFα.

33. The method of claim 18, wherein the method comprises administering to the subject a compound that inhibits 2-oxoglutarate dioxygenase enzyme activity.

34. The method of claim 21, wherein the method comprises administering to the subject a compound that inhibits HIF prolyl hydroxylase enzyme activity.

35. The method of claim 15, wherein the method further comprises administering to the subject a second compound.

36. The method of claim 35, wherein the second compound inhibits 2-oxoglutarate dioxygenase enzyme activity.

37. The method of claim 35, wherein the compound inhibits the activity of a first 2-oxoglutarate dioxygenase enzyme and the second compound inhibits the activity of a second 2-oxoglutarate dioxygenase enzyme.

38. The method of claim 35, wherein the second compound is selected from the group consisting of an ACE inhibitor (ACEI), angiotensin-II receptor blocker (ARB), diuretic, digoxin, statin, or carnitine.

39. The method of claim 32, wherein the compound stabilizes HIFα by specifically inhibiting hydroxylation of at least one amino acid residue in HIFα.

40. The method of claim 39, wherein the amino acid residue is selected from the group consisting of proline and asparagine.

41. A method for increasing expression of angiogenic factors in a subject, the method comprising stabilizing HIFα.

42. A method for increasing expression of glycolytic factors in a subject, the method comprising stabilizing HIFα.

43. A method for increasing expression of factors associated with oxidative stress in a subject, the method comprising stabilizing HIFα.

44. A method of treating a subject having a disorder associated with ischemic reperfusion injury, the method comprising stabilizing HIFα.

45. The method of claim 1, wherein the compound is selected from the group consisting of heterocyclic carboxamides, phenanthrolines, hydroxamates, and physiologically active salts and prodrugs derived therefrom.

46. The method of claim 45, wherein the heterocyclic carboxamides are selected from the group consisting of pyridine carboxamides, quinoline carboxamides, isoquinoline carboxamides, cinnoline carboxamides, and beta-carboline carboxamides.

47. The method of claim 1, wherein the compound is delivered in an oral formulation.

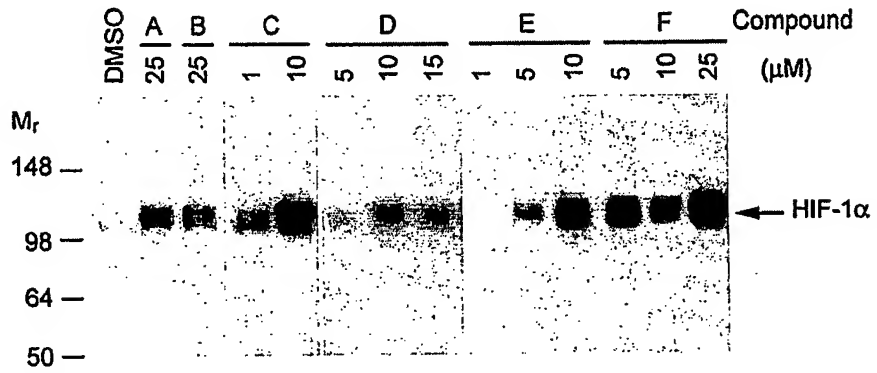
48. The method of claim 1, wherein the compound is delivered in a transdermal formulation.

49. A method of identifying a compound that stabilizes HIFα, the method comprising:

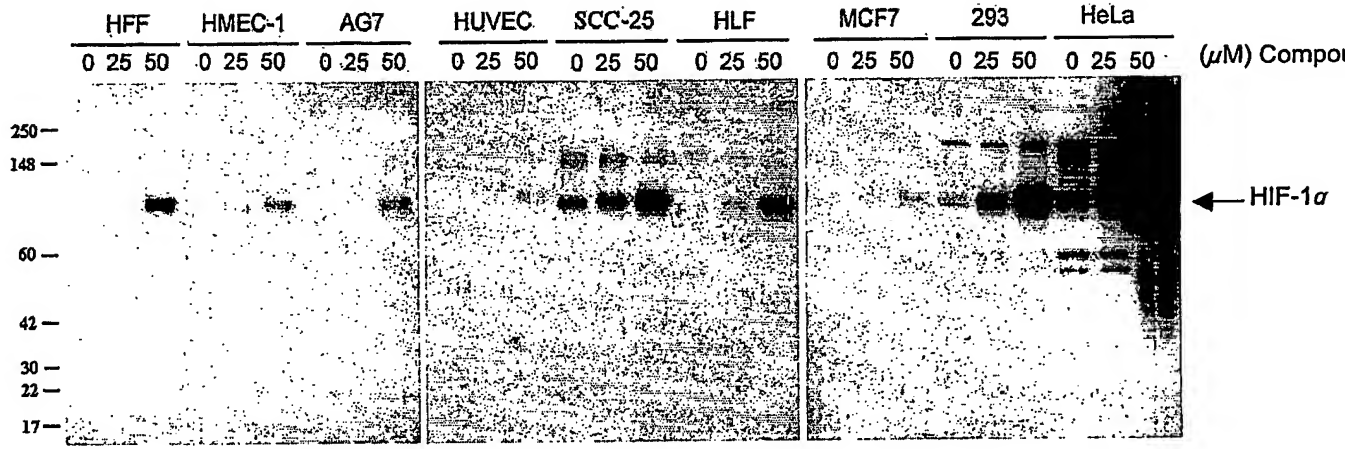
(a) administering a compound of interest to a subject or to a sample from a subject;

(b) measuring the HIFα level in the subject or in the sample; and

(c) comparing the HIFα level in the subject or in the sample to a standard level, wherein an increase in the HIFα level in the subject or the sample is indicative of a compound that stabilizes HIFα.


DMSO
A
B
C
D
E
F
Compound
25
25
1
10
5
10
15
1
5
10
5
10
25
(μM)
Mr
148
98
64
50
HIF-1α

A.


HFF
HMEC-1
AG7
HUVEC
SCC-25
HLF
MCF7
293
HeLa
0 25 50
(μM) Compound A
250
148
60
42
30
22
17
HIF-1α

B.

Figure 1

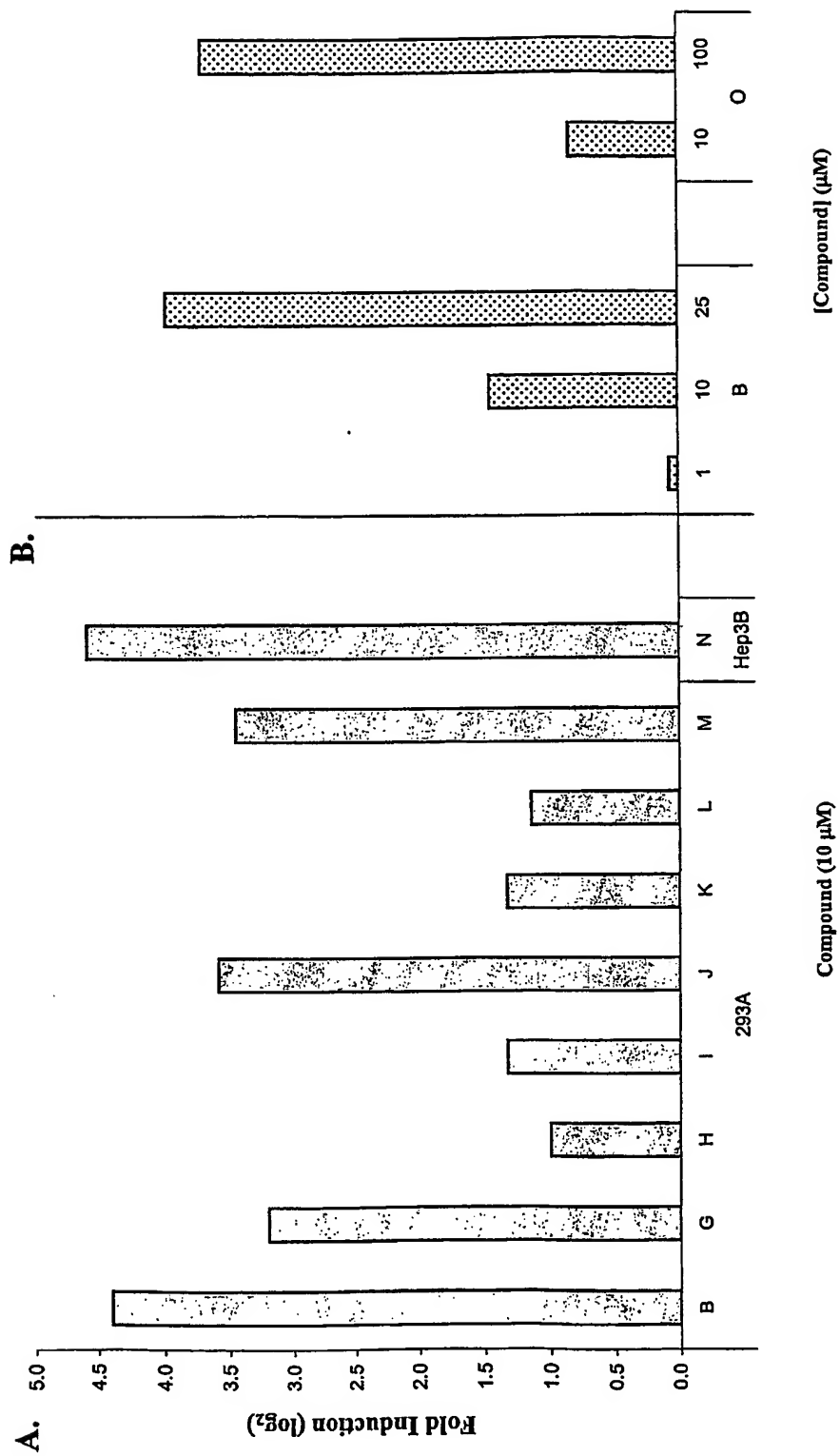



Figure 2

FP0600 PCT

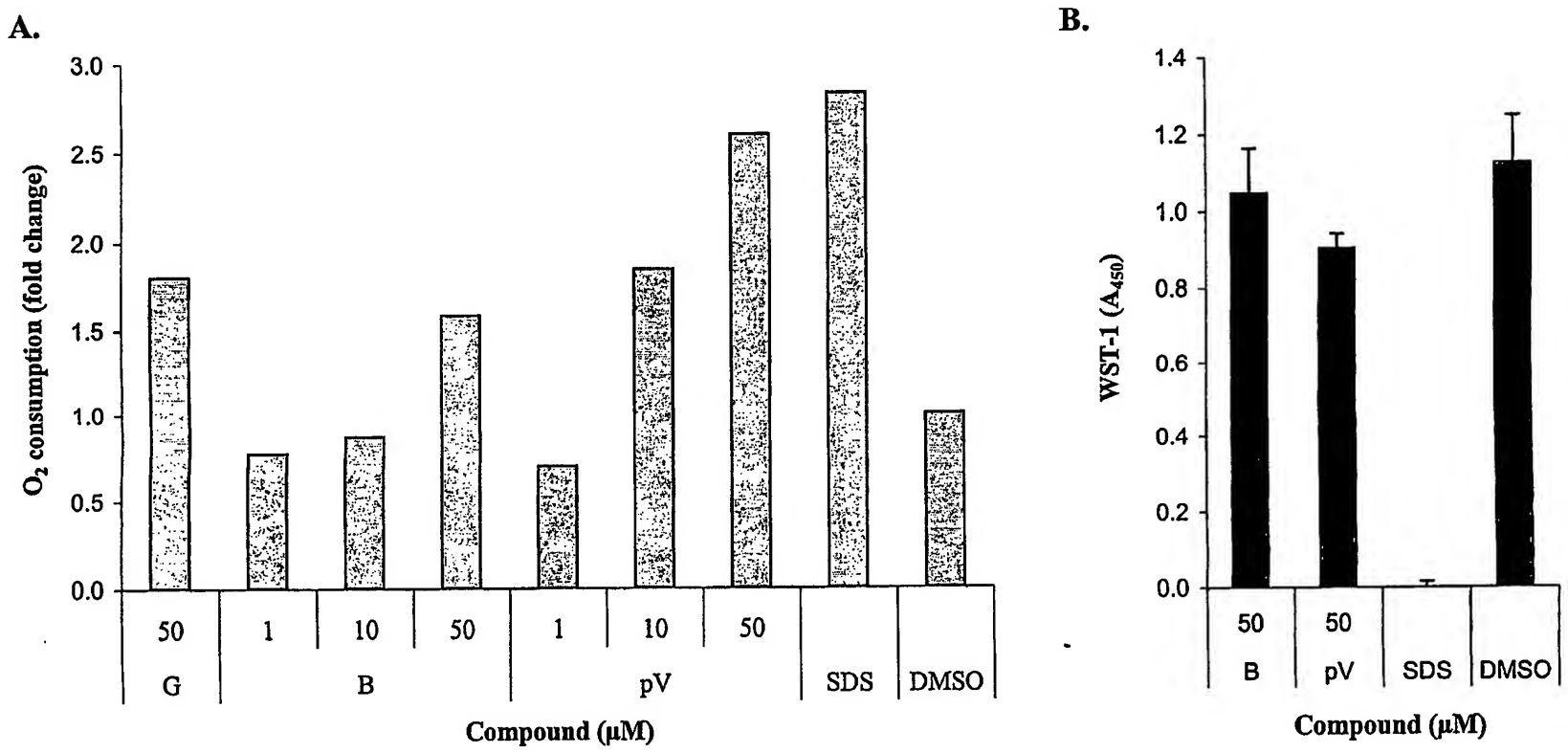
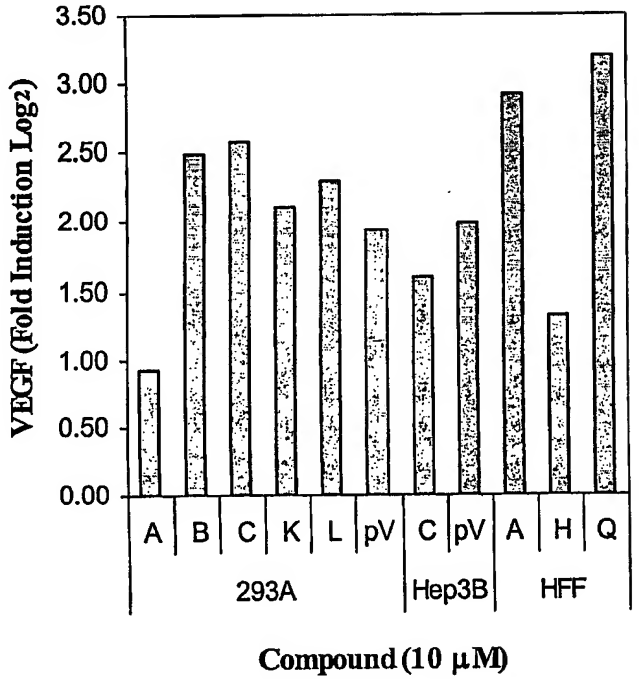
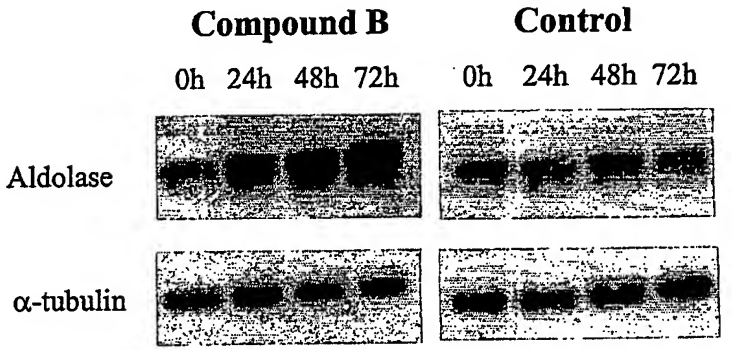

A.
$O_2$ consumption (fold change)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
50
1
10
50
1
10
50
G
B
pV
SDS
DMSO
Compound (μM)
B.
WST-1 ($A_{450}$)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
50
50
B
pV
SDS
DMSO
Compound (μM)

Figure 3

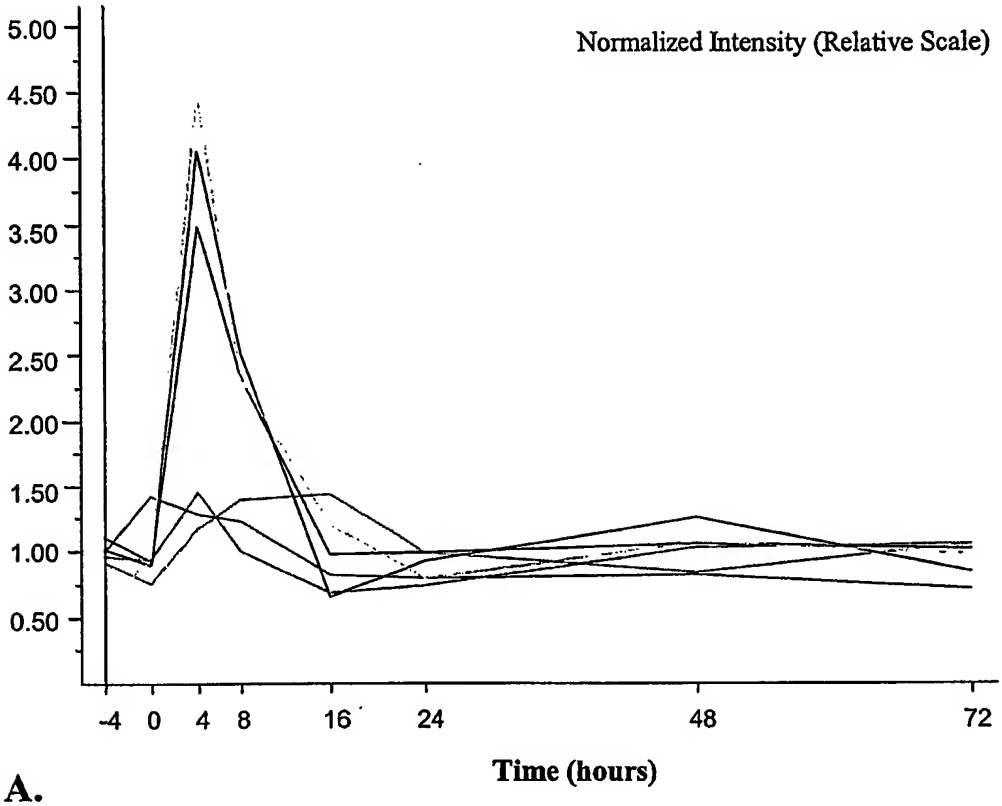

VEGF (Fold Induction Log2)
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
A
B
C
K
L
pV
C
pV
A
H
Q
293A
Hep3B
HFF
Compound (10 μM)

A.

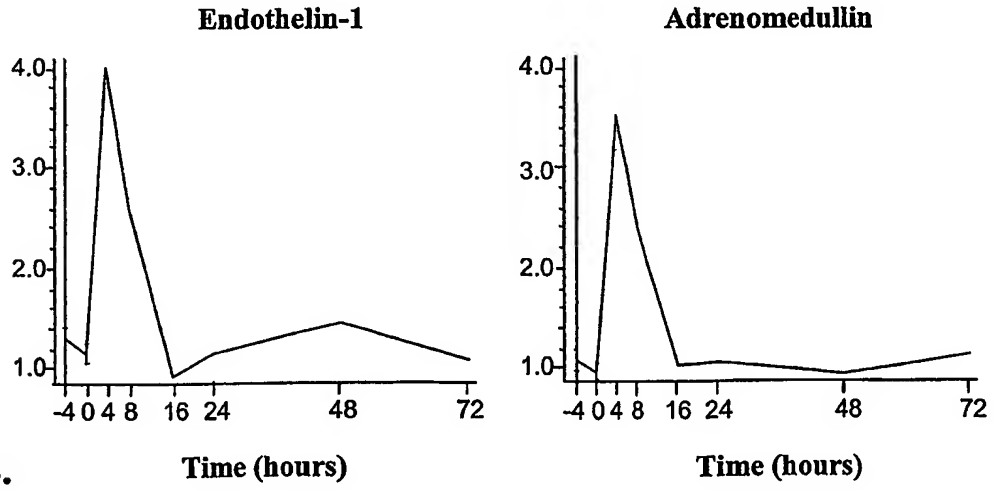

Compound B
Control
0h 24h 48h 72h
0h 24h 48h 72h
Aldolase
α-tubulin

B.

Figure 4


Normalized Intensity (Relative Scale)
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
-4 0 4 8 16 24 48 72
Time (hours)

A.


Endothelin-1
Adrenomedullin
4.0
3.0
2.0
1.0
-4 0 4 8 16 24 48 72
Time (hours)
Time (hours)

B.

Figure 5

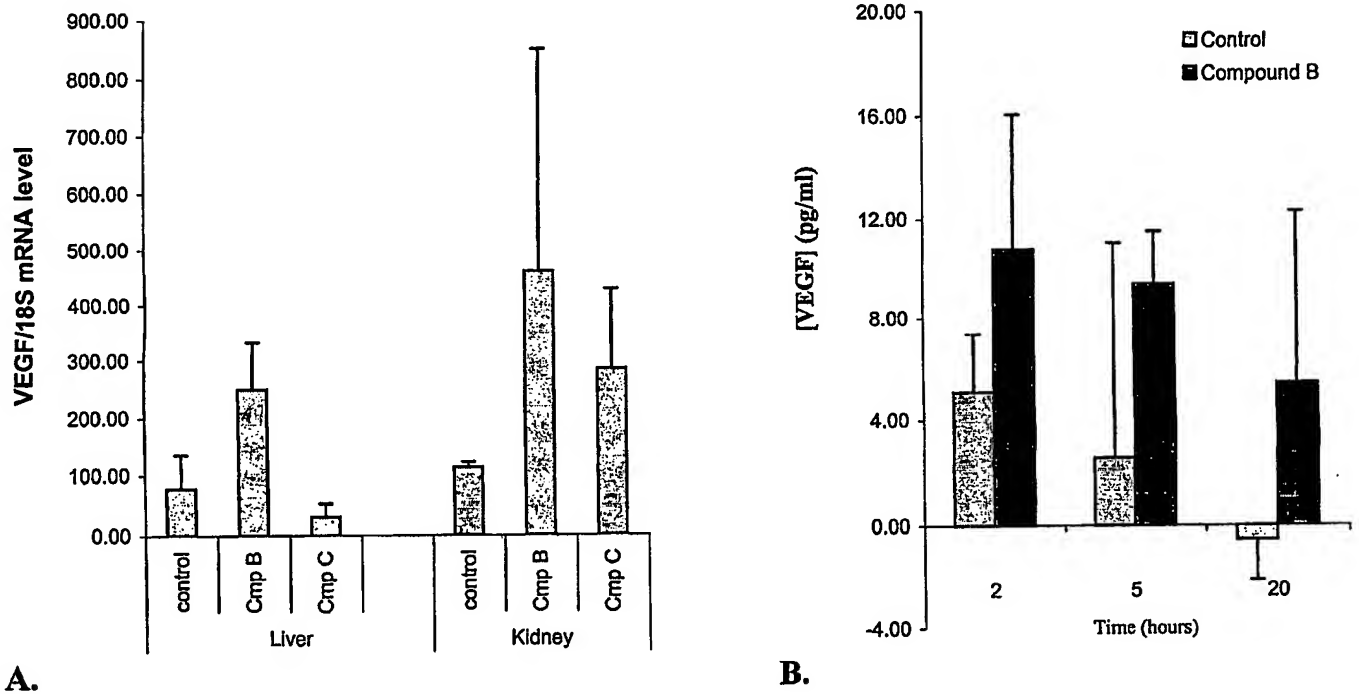
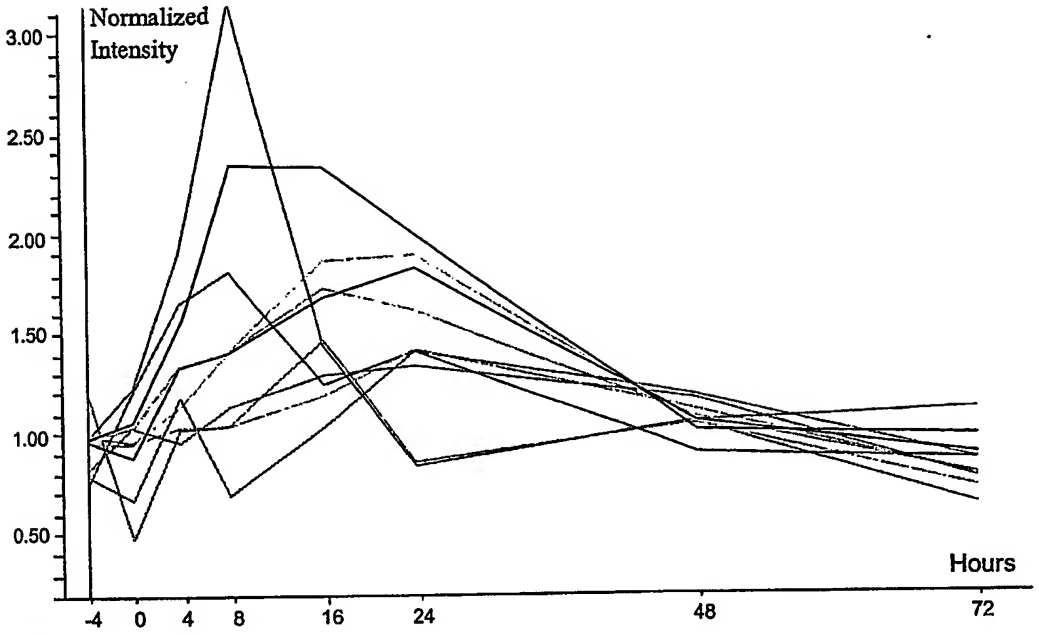
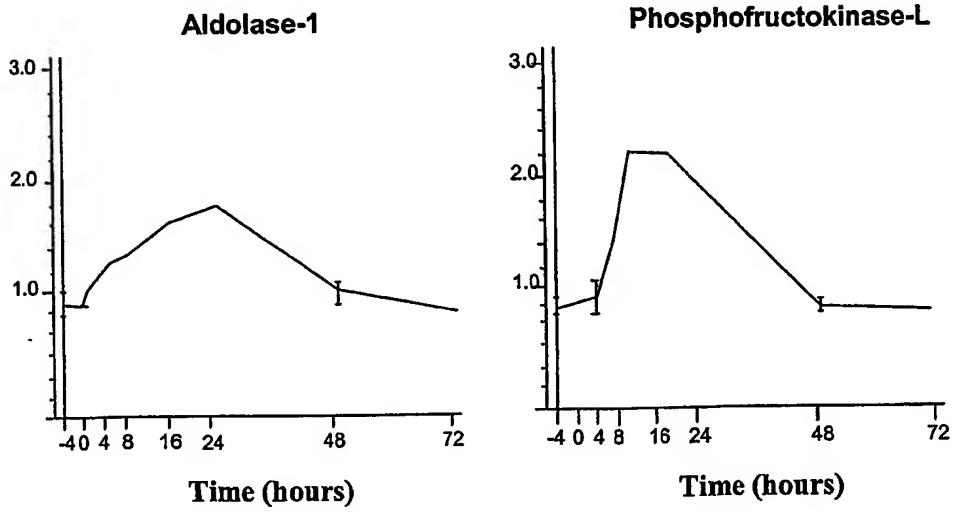

VEGF/18S mRNA level
900.00
800.00
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
control
Cmp B
Cmp C
Liver
control
Cmp B
Cmp C
Kidney
A.
[VEGF] (pg/ml)
20.00
16.00
12.00
8.00
4.00
0.00
-4.00
Control
Compound B
2
5
20
Time (hours)
B.

Figure 6




A.




B.

Error Bars: between-sample std. error

Figure 7

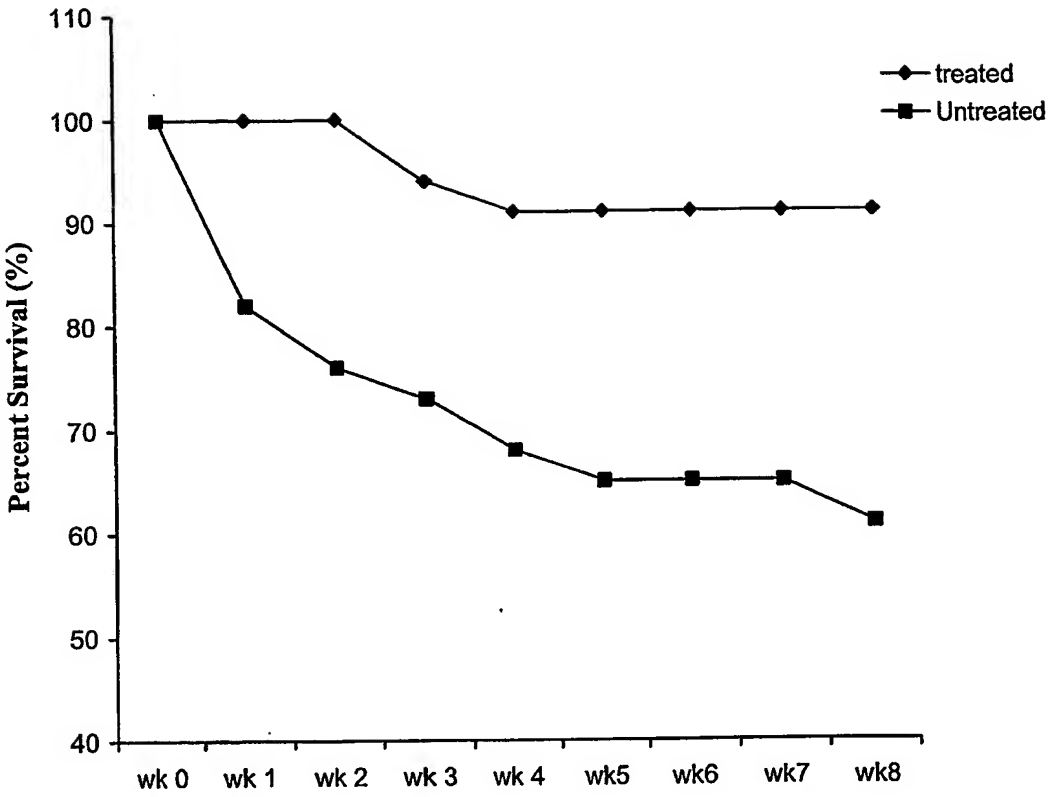
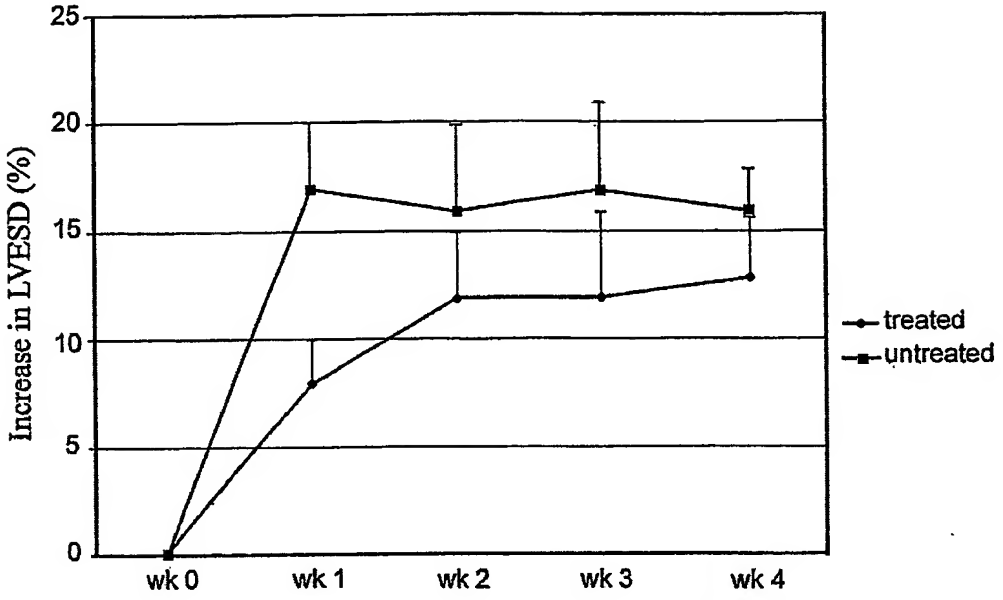
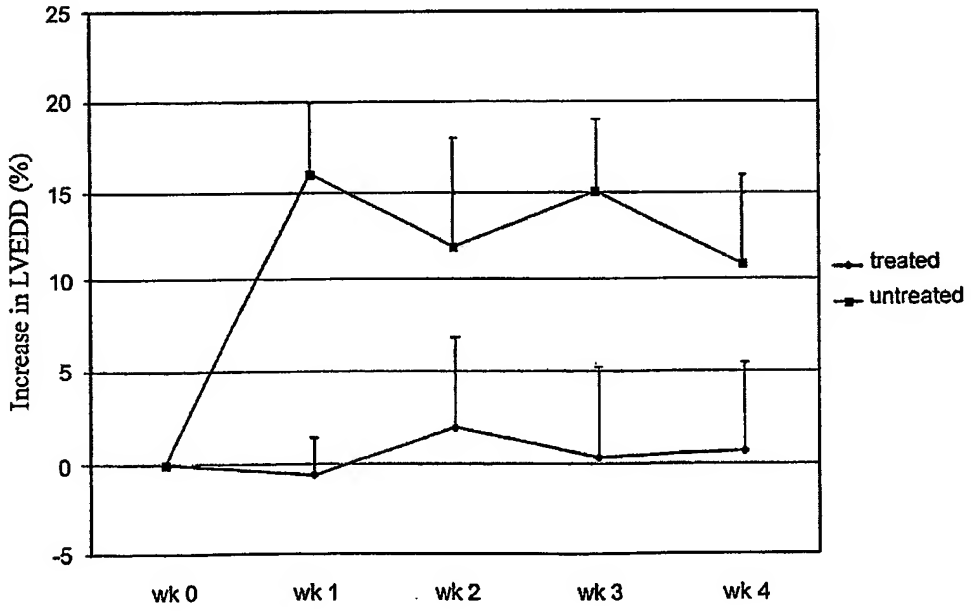

110
100
90
80
70
60
50
40
Percent Survival (%)
wk 0
wk 1
wk 2
wk 3
wk 4
wk5
wk6
wk7
wk8
treated
Untreated

Figure 8

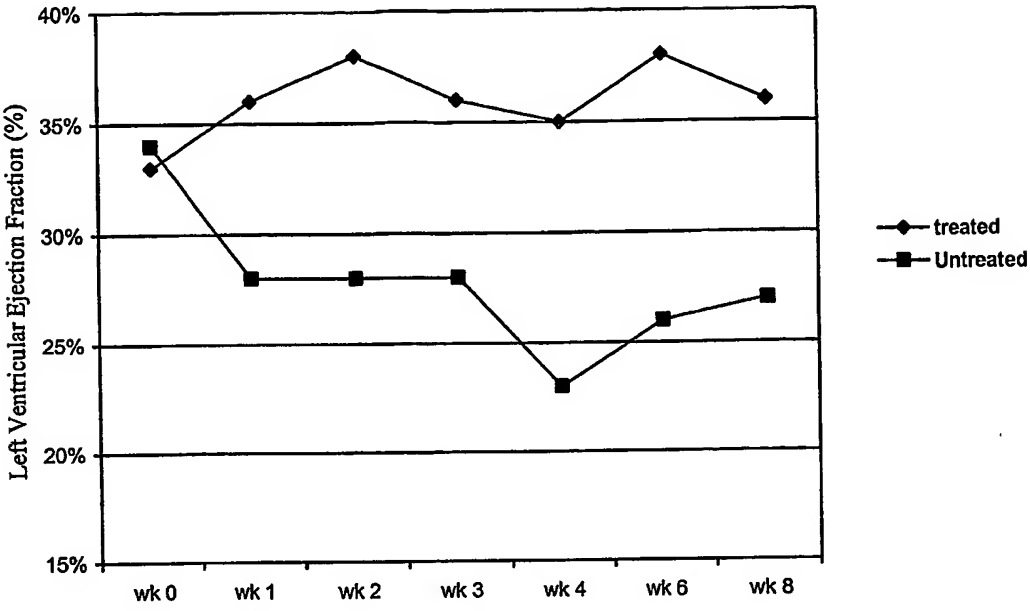

25
20
15
10
5
0
Increase in LVESD (%)
wk 0
wk 1
wk 2
wk 3
wk 4
treated
untreated

A.

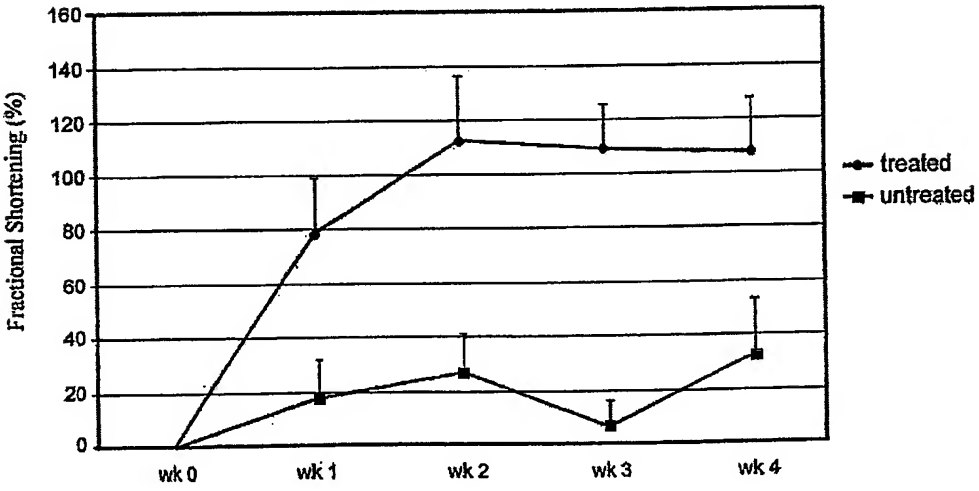

25
20
15
10
5
0
-5
Increase in LVEDD (%)
wk 0
wk 1
wk 2
wk 3
wk 4
treated
untreated

B.

Figure 9


40%
35%
30%
25%
20%
15%
Left Ventricular Ejection Fraction (%)
wk 0
wk 1
wk 2
wk 3
wk 4
wk 6
wk 8
treated
Untreated

A.


160
140
120
100
80
60
40
20
0
Fractional Shortening (%)
wk 0
wk 1
wk 2
wk 3
wk 4
treated
untreated

B.

Figure 10

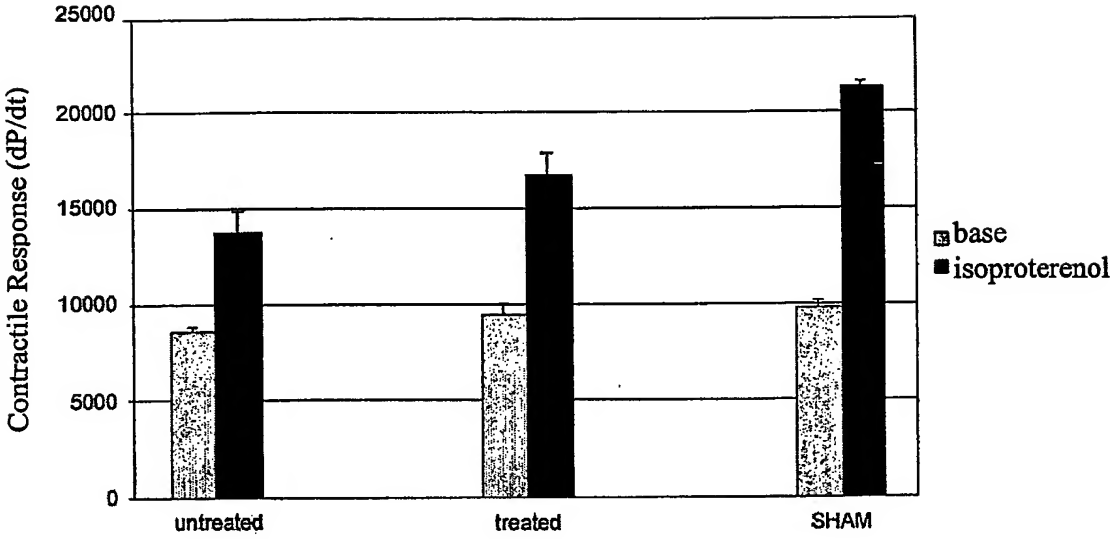
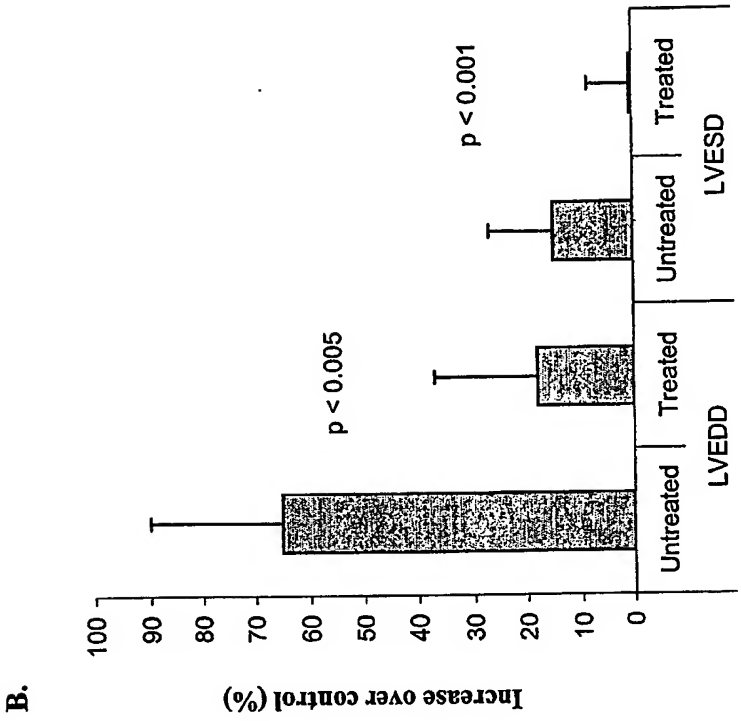

25000
20000
15000
10000
5000
0
Contractile Response (dP/dt)
untreated
treated
SHAM
base
isoproterenol

Figure 11


B.
Increase over control (%)
100
90
80
70
60
50
40
30
20
10
0
p < 0.005
p < 0.001
Untreated
Treated
Untreated
Treated
LVEDD
LVESD

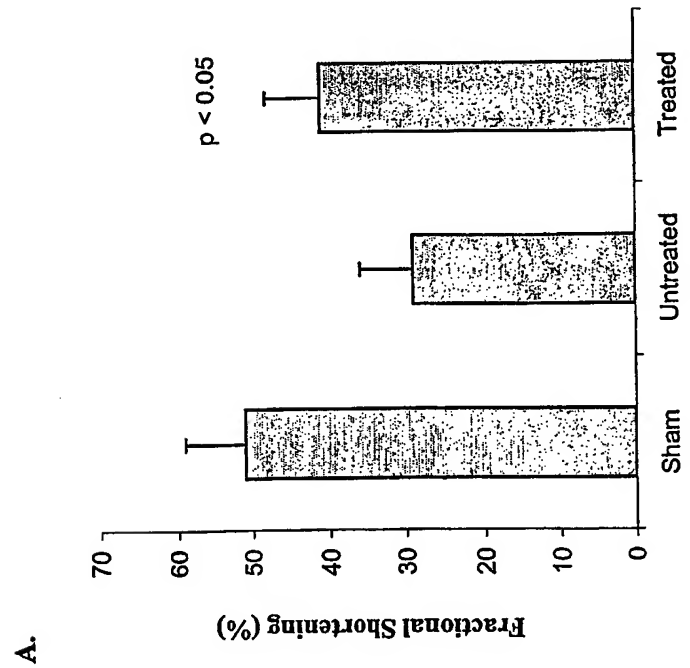

A.
Fractional Shortening (%)
70
60
50
40
30
20
10
0
p < 0.05
Sham
Untreated
Treated

Figure 12

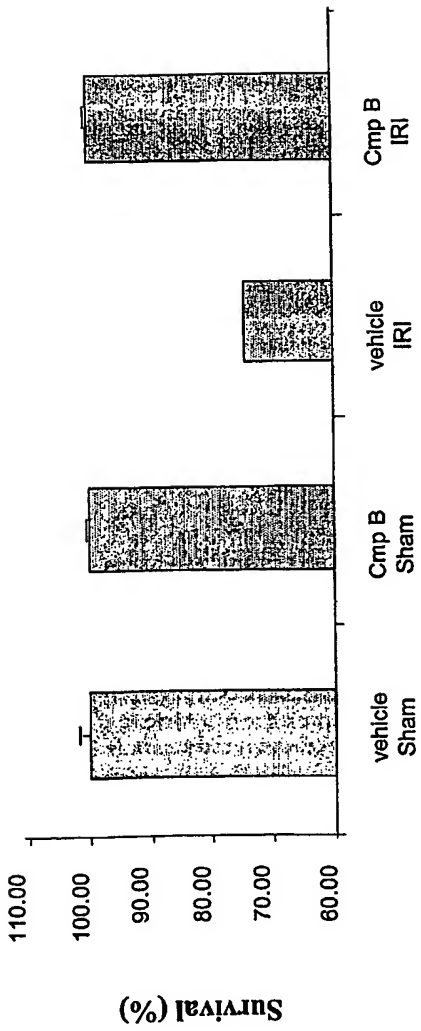
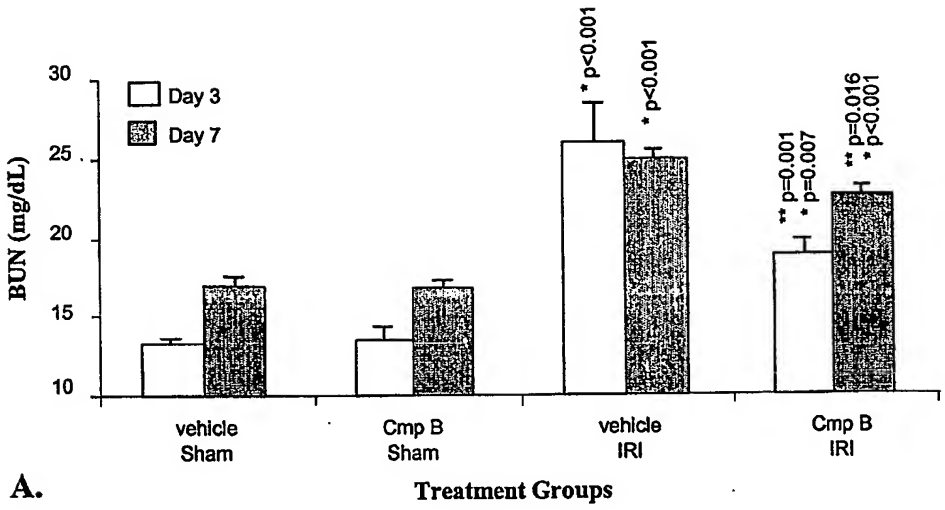
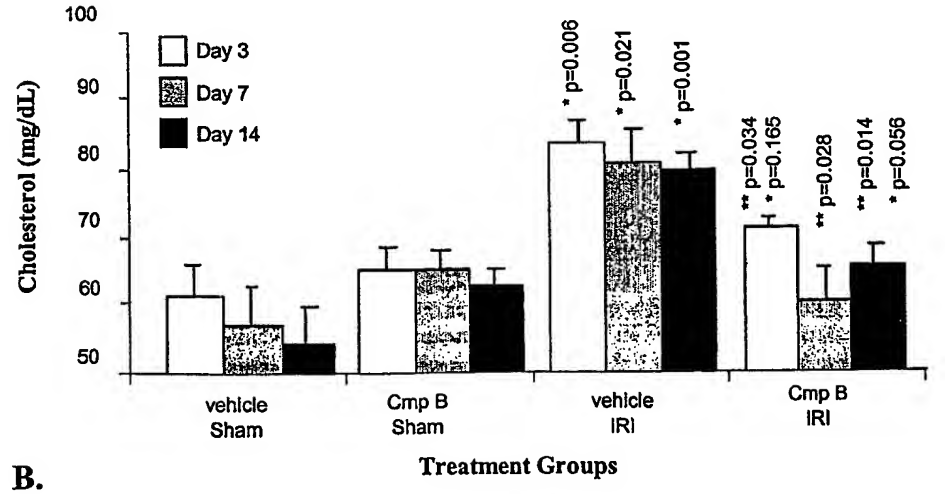

Survival (%)
110.00
100.00
90.00
80.00
70.00
60.00
vehicle Sham
Cmp B Sham
vehicle IRI
Cmp B IRI
Treatment Groups

Figure 13




A.




B.

* compared with CMC+Sham; ** compared with CMC+IRI, respectively

Figure 14

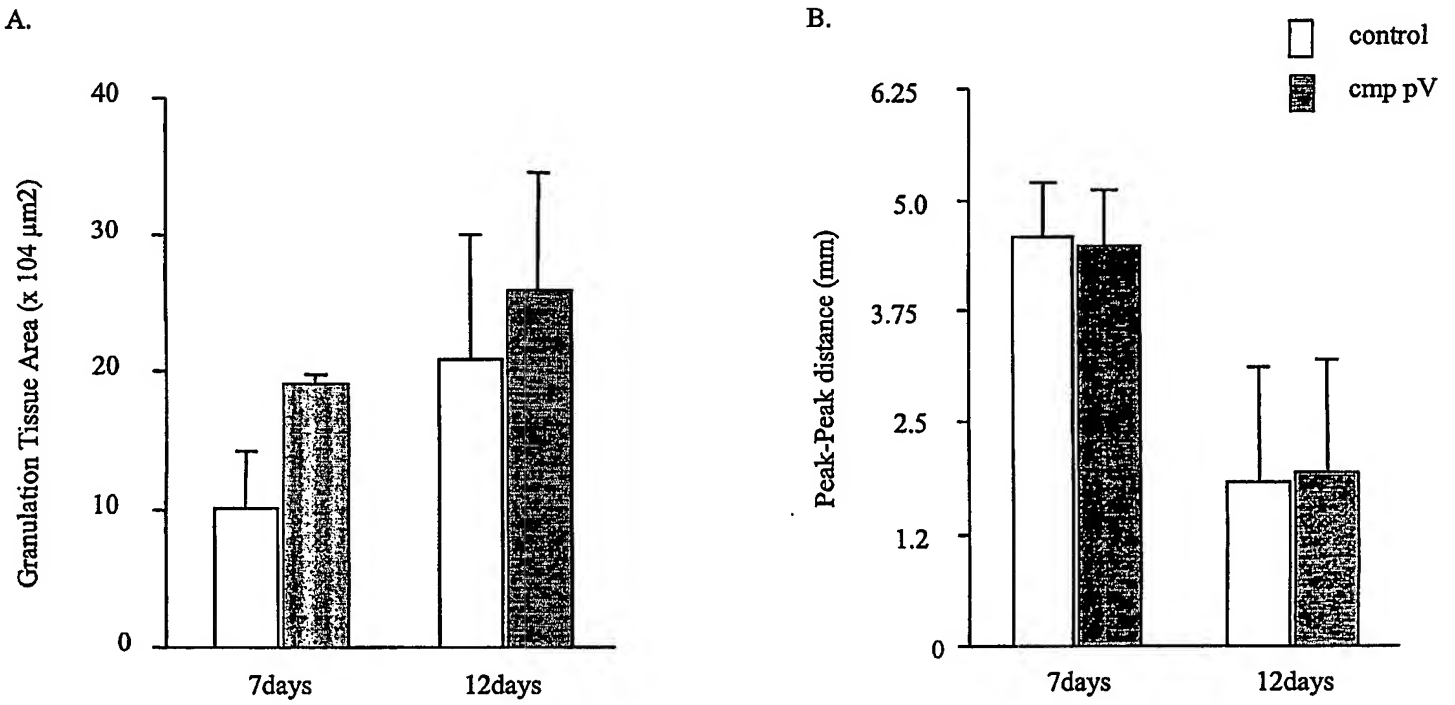

A.
Granulation Tissue Area (x 104 µm2)
40
30
20
10
0
7days
12days
B.
control
cmp pV
Peak-Peak distance (mm)
6.25
5.0
3.75
2.5
1.2
0
7days
12days

Figure 15

SEQUENCE LISTING

<110> Guenzler-Pukall, Volkmar
Neff, Thomas B.
Wang, Qingjian
Arend, Michael
Flippin, Lee A.
Melekhov, Alex

<120> STABILIZATION OF HYPOXIA INDUCIBLE FACTOR (HIF) ALPHA

<130> FP0600 PCT

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<151> 2001-12-06

<150> US 60/359,683
<151> 2002-02-25

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<151> 2002-01-16

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<212> DNA
<213> Mus musculus

<400> 1
gttgcaaggc gaggcagctt 20

<210> 2
<211> 21
<212> DNA
<213> Mus musculus

<400> 2
tgacgatgat ggcatggtgg t 21

<210> 3
<211> 22
<212> DNA
<213> Rattus norvegicus

<400> 3
taggcacggc gactaccatc ga 22

<210> 4
<211> 23
<212> DNA
<213> Rattus norvegicus

<400> 4
cggcggcttt ggtgactcta gat 23

<210> 5
<211> 19
<212> PRT
<213> synthetic peptide

<400> 5

Asp Leu Asp Leu Glu Ala Leu Ala Pro Tyr Ile Pro Ala Asp Asp Asp
1 5 10 15

Phe Gln Leu

US006124131A

# United States Patent [19]

Semenza

[11] Patent Number: 6,124,131

[45] Date of Patent: Sep. 26, 2000

[54] **MUTANT HYPOXIA INDUCIBLE FACTOR-1 HIF-1**

[75] Inventor: **Gregg L. Semenza**, Towson, Md.

[73] Assignee: **The Johns Hopkins University School of Medicine**, Baltimore, Md.

[21] Appl. No.: **09/148,547**

[22] Filed: **Aug. 25, 1998**

[51] **Int. Cl.**[7] ..... **C07K 16/00**; C12N 15/12; C12N 15/74; C12N 15/85

[52] **U.S. Cl.** ..... **435/325**; 435/69.1; 435/252.3; 435/320.1; 536/23.1; 536/23.5

[58] **Field of Search** ..... 435/320.1, 69.1, 435/325, 350, 7.1, 252.3; 530/350, 388.21; 424/93.1, 93.2, 93.21; 514/44; 536/23.1, 23.5

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*Primary Examiner*—John L. LeGuyader
*Assistant Examiner*—Carrie Stroup
*Attorney, Agent, or Firm*—Gray Cary Ware & Freidenrich LLP; Lisa A. Haile

[57] **ABSTRACT**

Substantially purified stable human hypoxia-inducible factor-1α (sHIF-1α) mutein is provided. Polynucleotides encoding stable human hypoxia-inducible factor-1α mutein are also provided. A method is provided for treating a hypoxia-related tissue damage in a subject by administering to the subject a therapeutically effective amount of a sHIF-1α mutein or a nucleotide sequence including an expression control sequence operatively linked to a polynucleotide encoding a stable hypoxia-inducible factor-1α mutein. Formulations are provided for the administration of stable human hypoxia inducible factor-1α (HIF-1α) polypeptide or a polynucleotide encoding stable human hypoxia inducible factor-1α (HIF-1α) to a patient having hypoxia-related tissue damage.

**18 Claims, 10 Drawing Sheets**

```
TAT GTG GAT AGT GAT ATG GTC AAT GAA TTC
tyr val asp ser asp met val asn glu phe
TTA GCT CCC TAT ATC CCA ATG GAT GAT GAC
leu ala pro tyr ile pro met asp asp asp
TTC CAG CAG ACT CAA ATA CAA GAA CCT ACT
phe gln gln thr gln ile gln glu pro thr
CCA TCT CCT ACC CAC ATA CAT AAA GAA ACT
pro ser pro thr his ile his lys glu thr
AAA TCT CAT CCA AGA AGC CCT AAC GTG TTA
lys ser his pro arg ser pro asn cal leu
ATG GAA CAT GAT GGT TCA CTT TTT CAA GCA
met glu his asp gly ser leu phe gln ala
GAA CAG AAT GGA ATG GAG CAA AAG ACA ATT
glu gln asn gly met glu gln lys thr ile
GTT AAT GCT CCT ATA CAA GGC AGC AGA AAC
val asn ala pro ile gln gly ser arg asn
AGTCTATTTATATTTTCTACATCTAATTTTAGAAGCCTGG

TTTTTGGTATTTAAACCATTGCATTGCAGTAGCATCATT
CATAGGCAGTTGAAAAATTTTTACACCTTTTTTTTCACA
TTAAGAAGAAATTTTTTTTGGCCTATGAAATTGTTAAAC
GGCATTTATTTGGATAAAATTCTCAATTCAGAGAAATCA
GTATAAAGATATTTTGAGCAGACTGTAAACAAGAAAAAA
TAATTTTAGAAGCATTATTTTAGGAATATATAGTTGTCA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

FIG. 1A

```
GAG CCT AAT AGT CCC AGT GAA TAT TGT TTT
glu pro asn ser pro ser glu tyr sys phe
ACT CAG GAC ACA GAT TTA GAC TTG GAG ATG
thr gln asp thr asp leu asp leu glu met
AGC GCA AGT CCT CAA AGC ACA GTT ACA GTA
ser ala ser pro gln ser thr val thr val
ATG GAA GAC ATT AAA ATA TTG ATT GCA TCT
met glu asp ile lys ile leu ile ala ser
GCA GGA AAA GGA GTC ATA GAA CAG ACA GAA
ala gly lys gly val ile glu gln thr glu
GCT TTG CAG AAT GCT CAG AGA AAG CGA AAA
ala leu gln asn ala gln arg lys arg lys
TGG AAA CGT GTA AAA GGA TGC AAA TCT AGT
trp lys arg val lys gly cys lys ser ser
TTA CCA CAG CTG ACC AGT TAT GAT TGT GAA
leu pro gln leu thr ser tyr asp cys glu
CATTCCTTTTTTTGGACACTGGTGGCTCACTACCTAAAGC

TGTTCTTTAATGCTGGATCACAGACAGCTCATTTTCTCAGT
AATATAATTTTTGTAAGAAGGCAGTAACCTTTCATCATGAT
AGTTACTCATGGAATATATTCTGCGTTTATAAAACTAGTTT
TACATAATATAGAAAGATATGCATATATCTAGAAGGTATGT
TGTAACTGATATTAAACCTAAATGTTCTGCCTACCCTGTTG
CTATTAACATCCTTTTTTTCATGTAGATTTCAATAATTGAG
ACTGTATTGTTTTGTTACATCAAATAAACATCTTCTGTGGA
```

FIG. 1B

```
TCC GAT GGA AGC ACT AGA CAA AGT TCA CCT
ser asp gly ser thr arg gln ser ser pro
GAA GAC ACA GAA GCA AAG AAC CCA TTT TCT
glu asp thr glu ala lys asn pro phe ser
CCA TTA GAA AGC AGT TCC GCA AGC CCT GAA
pro leu glu ser ser ser ala ser pro glu
GAT GAA TTA AAA ACA GTG ACA AAA GAC CGT
asp glu leu lys thr val thr lys asp arg
ACT CAA AGT CGG ACA GCC TCA CCA AAC AGA
thr gln ser arg thr ala ser pro asn arg
CCT GAG GAA GAA CTA AAT CCA AAG ATA CTA
pro glu glu glu leu asn pro lys ile leu
GAC GAT CAT GCA GCT ACT ACA TCA CTT TCT
asp asp his ala ala thr thr set leu ser
CTG CTG GGG CAA TCA ATG CAT GAA AGT GGA
leu leu gly gln ser met asp glu ser gly
TTG GAT CAA GTT AAC TGA GCTTTTTCTTAATTT
leu asp gln val asn OPA
CCCCTTTCTACTTAATTTACATTAATGCTCTTTTTTAGTA
GGAGTTTATCCCTTTTTCGAATTATTTTTAAGAAGATGCC
AGCCACAATTGCACAATATATTTTCTTAAAAAATACCAGC
AAATGCTGTATGGTTTATTATTTAAATGGGTAAAGCCATT
ACAATACCCTATGTAGTTGTGGAAGTTTATGCTAATATTG
TTTGCTCAAAATACAATGTTTGATTTTATGCACTTTGTCG
TTTTCATTCCTTTTGCTCTTTGTGGTTGGATCTAACACTA
```

FIG. 1C

```
1502 CCC CAG ATT CAG GAT CAG ACA CCT AGT CCT
 492 pro gln ile gln asp gln thr pro ser pro
1622 AAG TTG GAA TTG GTA GAA AAA CTT TTT GCT
 532 lys leu glu leu val glu lys leu phe ala
1742 TTC CAG TTA CGT TCC TTC GAT CAG TTG TCA
 572 phe gln leu arg ser phe asp gln leu ser
1862 GCT AAT GCC ACC ACT ACC ACT GCC ACC ACT
 612 ala asn ala thr thr thr thr ala thr thr
1982 ACT AGT GCC ACA TCA TCA CCA TAT AGA GAT
 652 thr ser ala thr ser ser pro tyr arg asp
2102 TCT GTC GCT TTG AGT CAA AGA ACT ACA GTT
 692 ser val ala leu ser gln arg thr thr val
2222 GTA GGA ATT GGA ACA TTA TTA CAG CAG CCA
 732 val gly ile gly thr leu leu gln gln pro
2342 ATT TTA ATA CCC TCT GAT TTA GCA TGT AGA
 772 ile leu ile pro ser asp leu ala cys arg
2462 CTA CTG CAG GGT GAA GAA TTA CTC AGA GCT
 812 leu leu gln gly glu glu leu leu arg ala
2605 CTACAATACTGCACAAACTTGGTTAGTTCAATTTTTGAT
2764 TTAAAAAATGCACCTTTTTATTTATTTATTTTTGGCTAG
2923 TTTTACATAAATAATAATGCTTTGCCAGCAGTACGTGGT
3082 CTGGAACATGACATTGTTAATCATATAATAATGATTCTT
3241 TCTGATGTTTCTATAGTCACTTTGCCAGCTCAAAAGAAA
3400 AAAATCATGCATTCTTAGCAAAATTGCCTAGTATGTTAA
3559 CAGTAAATATCTTGTTTTTTCTATGTACATTGTACAAAT
```

FIG. 1D

GCG GGC GCC GGC GGC GCG AAC GAC AAG AAA
glu gly ala gly gly ala asn asp lys lys
CAT CAG TTG CCA CTT CCA CAT AAT GTG AGT
his gln leu pro leu pro his asn val ser
GAT GAC ATG AAA GCA CAG ATG AAT TGC TTT
asp asp met lys ala gln met asn cys phe
TTA ACT CAG TTT GAA CTA ACT GGA CAC AGT
leu thr gln phe glu leu thr gly his ser
AAC ACA CAG CGA AGC TTT TTT CTC AGA ATG
asn thr gln arg ser phe phe leu arg met
GAT ACC AAC AGT AAC CAA CCT CAG TGT GGG
asp thr asn ser asn gln pro gln cys gly
TTC CTC AGT CGA CAC AGC CTG GAT ATG AAA
phe leu ser arg his ser leu asp met lys
TTG GAC TCT GAT CAT CTG ACC AAA ACT CAT
leu asp ser asp his leu thr lys thr his
ACT GTC ATA TAT AAC ACC AAG AAT TCT CAA
thr val ile tyr asn thr lys asn ser gln
AAA CCG GTT GAA TCT TCA GAT ATG AAA ATG
lys pro val glu ser ser asp met lys met
CCA GCC GCT GGA GAC ACA ATC ATA TCT TTA
pro ala ala gly asp thr ile ile ser leu
AAA TTA CAG AAT ATA AAT TTG GCA ATG TCT
lys leu gln asn ile asn leu ala met ser
CCA GAG TCA CTG GAA CTT TCT TTT ACC ATG
pro glu ser leu glu leu ser phe thr met

FIG. 1E

```
                GTGAAGACATCGCGGGGACCGATTCACC ATG
                                             met
AAA GAA TCT GAA GTT TTT TAT GAG CTT GCT
lys glu ser glu val phe tyr glu leu ala
CTT CTG GAT GCT GGT GAT TTG GAT ATT GAA
leu leu asp ala gly asp leu asp ile glu
ATT TCT GAT AAT GTG AAC AAA TAC ATG GGA
ile ser asp asn val asn lys tyr met gly
AAT GGC CTT GTG AAA AAG GGT AAA GAA CAA
asn gly leu yal lys lys gly lys glu glu
TTG CAC TGC ACA GGC CAC ATT CAC GTA TAT
leu his cys thr gly his ile his val tyr
AAT ATT GAA ATT CCT TTA GAT AGC AAG ACT
asn ile glu ile pro leu asp ser lys thr
GGC CGC TCA ATT TAT GAA TAT TAT CAT GCT
gly arg ser ile tyr glu tyr tyr his ala
GGT GGA TAT GTC TGG GTT GAA ACT CAA GCA
gly gly tyr val trp val glu thr gln ala
TTC TCC CTT CAA CAA ACA GAA TGT GTC CTT
phe ser leu gln gln thr glu cys val leu
AAG GAA CCT GAT GCT TTA ACT TTG CTG GCC
lys glu pro asp ala leu thr leu leu ala
AAT GAT GTA ATG CTC CCC TCA CCC AAC GAA
asn asp val met leu pro ser pro asn glu
CAA GAA GTT GCA TTA AAA TTA GAA CCA AAT
gln glu val ala leu lys leu glu pro asn
```

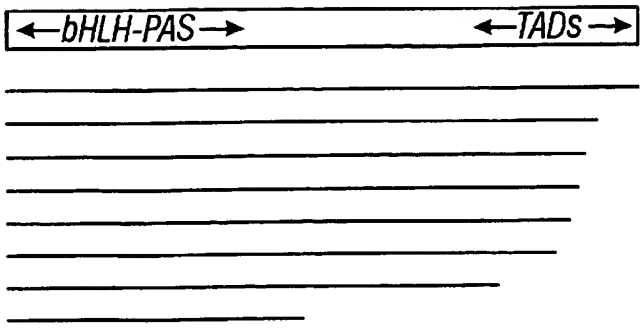
FIG. 1F

```
TCT CGA GAT GCA GCC AGA TCT CGG CGA AGT
ser arg asp ala ala arg ser arg arg ser
CTT ACC ATC AGC TAT TTG CGT GTG AGG AAA
leu thr ile ser tyr leu arg val arg lys
GTT CTC ACA GAT GAT GGT GAC ATG ATT TAC
val leu thr asp asp gly asp met ile tyr
GAG GAA ATG AGA GAA ATG CTT ACA CAC AGA
glu glu met arg glu met leu thr his arg
ATG AAC ATA AAG TCT GCA ACA TGG AAG GTA
met asn ile lys ser ala thr trp lys val
CTG ATT TGT GAA CCC ATT CCT CAC CCA TCA
leu ile cys glu pro ile pro his pro ser
TTG ATG GGA TAT GAG CCA GAA GAA CTT TTA
leu met gly tyr glu pro glu glu leu leu
ACA GGA CAG TAC AGG ATG CTT GCC AAA AGA
thr gly gln tyr arg met leu ala lys arg
GTG AGT GGT ATT ATT CAG CAC GAC TTG ATT
val ser gly ile ile gln his asp leu ile
GAT ACA AGT AGC CTC TTT GAC AAA CTT AAG
asp thr ser ser leu phe asp lys leu lys
GAC CAG CAA CTT GAG GAA GTA CCA TTA TAT
asp gln gln leu glu glu val pro leu tyr
CTT CGA AGT AGT GCT GAC CCT GCA CTC AAT
leu arg ser ser ala asp pro ala leu asn
```

FIG. 1G

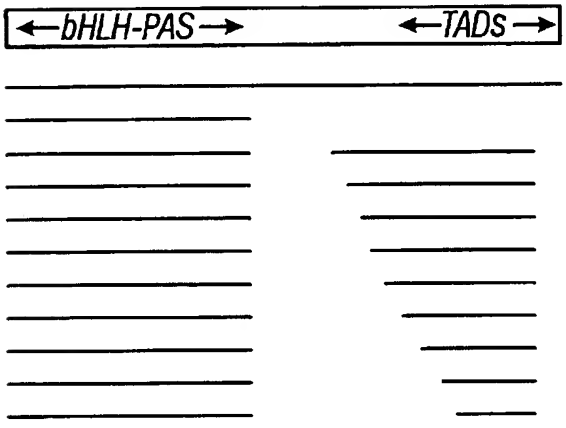
```
   1
   1
  62 AAG ATA AGT TCT GAA CGT CGA AAA GAA AAG
  12 lys ile ser ser glu arg arg lys glu lys
 182 TCG CAT CTT GAT AAG GCC TCT GTG ATG AGG
  52 ser his leu asp lys ala ser val met arg
 302 TAT TTG AAA GCC TTG GAT GGT TTT GTT ATG
  92 tyr leu lys ala leu asp gly phe val met
 422 GTG TTT GAT TTT ACT CAT CCA TGT GAC CAT
 132 val phe asp phe thr his pro cys asp his
 542 AAG TGT ACC CTA ACT AGC CGA GGA AGA ACT
 172 lys cys thr leu thr ser arg gly arg thr
 662 TAT AAG AAA CCA CCT ATG ACC TGC TTG GTG
 212 tyr lys lys pro pro met thr cys leu val
 782 TTT TCT TAT TGT GAT GAA AGA ATT ACC GAA
 252 phe ser tyr cys asp glu arg ile thr glu
 902 CAT GAT ATG TTT ACT AAA GGA CAA GTC ACC
 292 his asp met phe thr lys gly gln val thr
1022 CCA CAG TGC ATT GTA TGT GTG AAT TAC GTT
 332 pro gln cys ile val cys val asn tyr val
1142 ACT CAG CTA TTC ACC AAA GTT GAA TCA GAA
 372 thr gln leu phe thr lys val glu ser glu
1262 GAT TTT GGC AGC AAC GAC ACA GAA ACT GAT
 412 asp phe gly ser asn asp thr glu thr asp
1382 CCA TTA CCC ACC GCT GAA ACG CCA AAG CCA
 452 pro leu pro thr ala glu thr pro lys pro
```

FIG. 1H




| RESIDUES | REGULATION |
|---|---|
| 1-826 | + |
| 1-754 | + |
| 1-729 | + |
| 1-726 | + |
| 1-703 | - |
| 1-681 | - |
| 1-608 | - |
| 1-390 | - |

FIG. 2




| RESIDUES | REGULATION Wt | REGULATION mut |
|---|---|---|
| 1-826 | + | + |
| 1-390 | - | |
| 1-391, 429-826 | + | - |
| 1-391, 469-826 | + | - |
| 1-391, 494-826 | + | - |
| 1-391, 508-826 | + | - |
| 1-391, 512-826 | + | - |
| 1-391, 517-826 | + | - |
| 1-391, 521-826 | - | |
| 1-391, 549-826 | - | |
| 1-391, 576-826 | - | |

FIG. 3

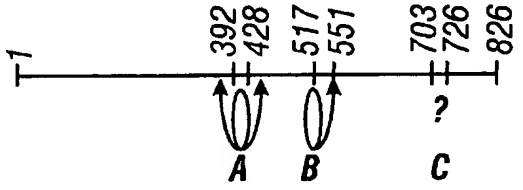



FIG. 4

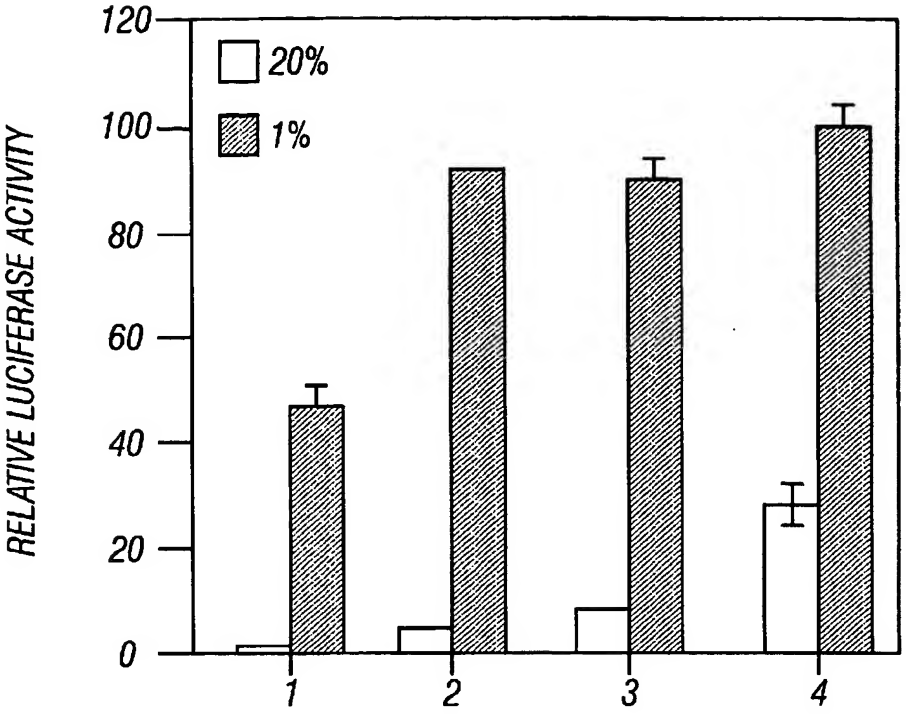

RELATIVE LUCIFERASE ACTIVITY
120
100
80
60
40
20
0
20%
1%
1
2
3
4

FIG. 5

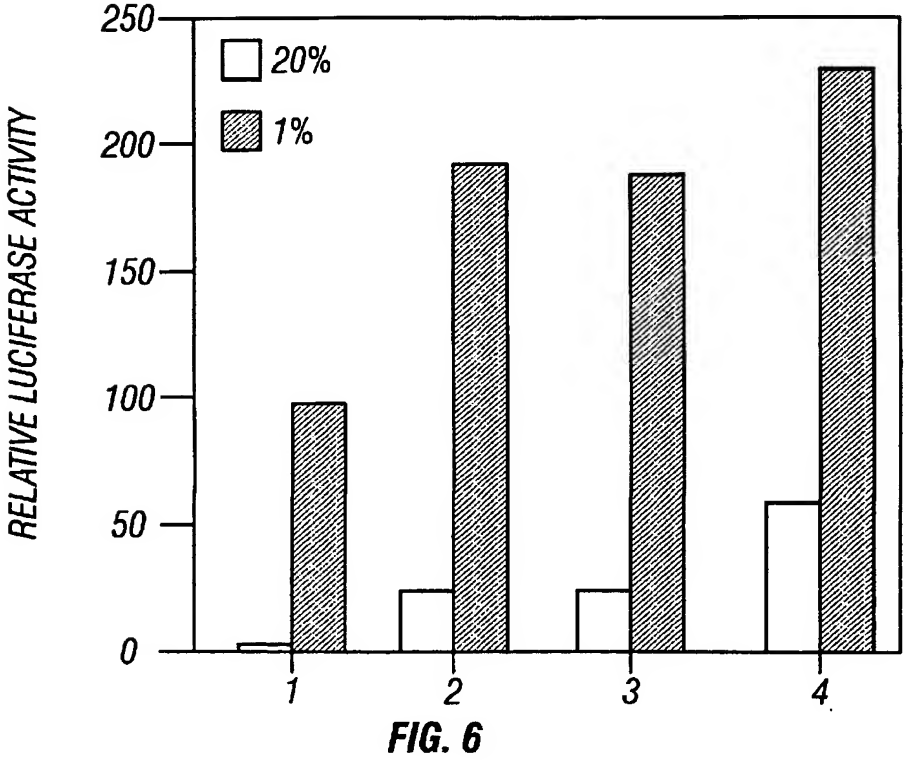

RELATIVE LUCIFERASE ACTIVITY
250
200
150
100
50
0
20%
1%
1
2
3
4

FIG. 6

# MUTANT HYPOXIA INDUCIBLE FACTOR-1 HIF-1

## STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

This invention was made in part with funds from the National Heart, Lung, and Blood Institute, grant number 1R01-HL55338. The government may have certain rights in this invention.

## FIELD OF THE INVENTION

This invention relates generally to hypoxia-related proteins and more specifically to DNA-binding proteins which are stable under non-hypoxic and hypoxic conditions.

## BACKGROUND OF THE INVENTION

Mammals require molecular oxygen ($O_2$) for essential metabolic processes including oxidative phosphorylation in which $O_2$ serves as electron acceptor during ATP formation. Systemic, local, and intracellular homeostatic responses elicited by hypoxia (the state in which $O_2$ demand exceeds supply) include erythropoiesis by individuals who are anemic or at high altitude (Jelkmann, *Physiol. Rev.* 72:449–489, 1992), neovascularization in ischemic myocardium (White et al., *Circ. Res.* 71:1490–1500, 1992), and glycolysis in cells cultured at reduced $O_2$ tension (Wolfe et al., *Eur. J. Biochem.* 135:405–412, 1983). These adaptive responses either increase $O_2$ delivery or activate alternate metabolic pathways that do not require $O_2$. Hypoxia-inducible gene products that participate in these responses include erythropoietin (EPO) (reviewed in Semenza, *Hematol. Oncol. Clinics N. Amer.* 8:863–884, 1994), vascular endothelial growth factor (Shweiki et al., *Nature* 359:843–845, 1992; Banai et al., *Cardiovasc. Res.* 28:1176–1179, 1994; Goldberg & Schneider, *J. Biol. Chem.* 269:4355–4359, 1994), and glycolytic enzymes (Firth et al., *Proc. Natl. Acad. Sci. USA* 91:6496–6500, 1994; Semenza et al., *J. Biol. Chem.* 269:23757–23763, 1994).

The molecular mechanisms that mediate genetic responses to hypoxia have been extensively investigated for the EPO gene, which encodes a growth factor that regulates erythropoiesis and thus blood $O_2$-carrying capacity (Jelkmann, 1992, supra; Semenza, 1994, supra). Cis-acting DNA sequences required for transcriptional activation in response to hypoxia were identified in the EPO 3'-flanking region and a trans-acting factor that binds to the enhancer, hypoxia-inducible factor 1 (HIF-1), fulfilled criteria for a physiological regulator of EPO transcription. In particular, inducers of EPO expression (1% $O_2$, cobalt chloride [$CoCl_2$], and desferrioxamine [DFX]) also induced HIF-1 DNA binding activity with similar kinetics. In addition, inhibitors of EPO expression (actinomycin D, cycloheximide, and 2-aminopurine) blocked induction of HIF-1 activity. Furthermore, mutations in the EPO 3'-flanking region that eliminated HIF-1 binding also eliminated enhancer function (Semenza, 1994, supra). These results also support the hypothesis that $O_2$ tension is sensed by a hemoprotein (Goldberg et al., *Science* 242:1412–1415, 1988) and that a signal transduction pathway requiring ongoing transcription, translation, and protein phosphorylation participates in the induction of HIF-1 DNA-binding activity and EPO transcription in hypoxic cells (Semenza, 1994, supra).

EPO expression is cell type specific, but induction of HIF-1 activity by 1% $O_2$, $CoCl_2$, or DFX was detected in many mammalian cell lines (Wang & Semenza, *Proc. Natl. Acad. Sci. USA* 90:4304–4308, 1993). The EPO enhancer directed hypoxia-inducible transcription of reporter genes transfected into non-EPO-producing cells (Wang & Semenza, 1993, supra; Maxwell et al., *Proc. Natl. Acad. Sci. USA* 90:2423–2427, 1993). RNAs encoding several glycolytic enzymes were induced by 1% $O_2$, $CoCl_2$, or DFX in EPO-producing Hep3B or nonproducing HeLa cells whereas cycloheximide blocked their induction and glycolytic gene sequences containing HIF-1 binding sites mediated hypoxia-inducible transcription in transfection assays (Firth et al., 1994, supra; Semenza et al., 1994, supra). These experiments support the role of HIF-1 in activating homeostatic responses to hypoxia.

Hypoxia inducible factor-1 (HIF-1) is a mammalian transcription factor expressed uniquely in response to physiologically relevant levels of hypoxia (Wang, G. L., et al., *Proc. Natl. Acad. Sci. USA* 92:5510–5514, 1995; Wang, G. L., and Semenza, G. L., *J. Biol. Chem.* 270:1230–1237, 1995). HIF-1 is a basic helix loop-helix protein that binds to cis-acting hypoxia-responsive elements of genes induced by hypoxia (Wang, G. L., and Semenza, G. L., *Curr. Opin. Hematol.* 3:156–162, 1992; Jiang, B. H., et al., *J. Biol. Chem.* 272:19253–19260, 1997). The genes that are activated by HIF-1 in cells subjected to hypoxia include EPO, vascular endothelial growth hormone (VEGF), heme oxygenase-1, inducible nitric oxide synthase, and glycolytic enzymes aldolase A, enolase 1, lactate dehydrogenase A, phosphofructokinase I, and phosphoglycerate kinase 1 (Semenza, G. L., et al., *Kid. Int.* 51:553–555, 1997). HIF-1 DNA binding activity and HIF-1 protein concentration increases exponentially as cells are subjected to decreasing $O_2$ concentrations (Jiang, B. H., et al., *Am J. Physiol.* 271:C1172–C1180, 1996).

HIF-1 is a heterodimer of two subunits, HIF-1α and HIF-1β. The HIF-1α subunit is unique to HIF-1, whereas HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator, ARNT) can dimerize with other proteins. The concentration of HIF-1α and HIF-1β RNA and HIF-1α and HIF-1β polypeptide increases in cells exposed to hypoxic conditions (Wiener, C. M., et al., *Biochem. Biophys. Res. Commun.* 225:485–488, 1996).

Structural analysis of HIF-1α revealed that dimerization requires two domains, termed HLH and PAS. DNA binding is mediated by a basic domain (Semenza, G. L., et al., *Kid. Int.* 51:553–555, 1997). Two transactivation domains are contained in HIF-1α, located between amino acids 531 and 826. The minimal transactivation domains are at amino acid residues 531–575 and 786–826 (Jiang, B. H., et al., 1997, supra; Semenza, G. L., et al., 1997, supra). Amino acids 1–166 are key in mediating heterodimerization with HIF-1β (ARNT), but amino acids 1–390 are required for optimal DNA binding. In addition, deletion of the carboxy terminus of HIF-1α (amino acids 391–826) decreased the ability of HIF-1 to activate transcription. However, HIF-1α (1–390) was expressed at high levels in both hypoxic and non-hypoxic cells in contrast to full-length HIF-1α (1–826) which was expressed at much higher levels in hypoxic relative to non-hypoxic cells (Jiang, B.-H., et al., *J. Biol. Chem.* 271:17771–17778, 1996).

## SUMMARY OF THE INVENTION

This invention is based on the discovery and isolation of unique variant forms of HIF-1α polypeptide that are stable under hypoxic and nonhypoxic conditions.

In one embodiment, the invention provides a substantially purified polypeptide having a sequence as set forth in SEQ

ID NO:22 wherein amino acids 392 to 428 are deleted therefrom, amino acid 551 is changed from a serine to any other amino acid, and amino acid 552 is changed from a threonine to any other amino acid. Isolated polynucleotides encoding such a polypeptide as well as antibodies which preferentially bind this polypeptide are also provided in a particular embodiment, serine 551 is changed to glycine and threonine 552 to alanine.

In one embodiment, a method is provided for treating a hypoxia-related tissue damage in a subject, by administering to the subject a therapeutically effective amount of a nucleotide sequence comprising an expression control sequence operatively linked to a polynucleotide encoding a polypeptide having a sequence as set forth in SEQ ID NO:1, wherein amino acids 392 to 428 are deleted therefrom, amino acid 551 is changed from a serine to any other amino acid, and amino acid 552 is changed from a threonine to any other amino acid.

In another embodiment, the invention provides a method of treating a hypoxia-related tissue damage in a subject by administering to the subject a therapeutically effective amount of a polypeptide having a sequence as set forth in SEQ ID NO:1, wherein amino acids 392 to 428 are deleted therefrom, amino acid 551 is changed from a serine to any other amino acid, and amino acid 552 is changed from a threonine to any other amino acid.

In a further embodiment, the invention provides a formulation for administration of stable human hypoxia inducible factor-1α (HIF-1α) polypeptide to a patient having hypoxia related tissue damage. The method includes a substantially pure polypeptide having a sequence as set forth in SEQ ID NO:1, wherein amino acids 392 to 428 are deleted therefrom, amino acid 551 is changed from a serine to any other amino acid, and amino acid 552 is changed from a threonine to any other amino acid; and a pharmaceutically acceptable carrier.

The invention also provides a formulation for administration of a polynucleotide encoding stable human hypoxia inducible factor-1α (HIF-1α) to a patient having hypoxia related tissue damage, including a therapeutically effective amount of a nucleic acid sequence comprising an expression control sequence operatively linked to a polynucleotide encoding a polypeptide having a sequence as set forth in SEQ ID NO:1, wherein amino acids 392 to 428 are deleted therefrom, amino acid 551 is changed from a serine to any other amino acid, and amino acid 552 is changed from a threonine to any other amino acid; and a pharmaceutically acceptable carrier.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is the amino acid sequence (SEQ ID NO:2) and nucleotide sequence (SEQ ID NO:1) of wild-type HIF-1α.

FIG. 2 shows an analysis of the effect of carboxyl-terminal deletions on the regulated expression of HIF-1α.

FIG. 3 shows an analysis of the effect of internal deletions on regulated expression of HIF-1α polypeptide. Oxygen regulation of the HIF-1α polypeptide containing the indicated internal deletion is shown in the "wt" column, where a "+" indicates that the polypeptide is regulated, and is therefore unstable under non-hypoxic conditions. Each of the indicated internal deletions in HIF-1α has been combined with a double point mutation (a serine to glycine mutation at amino acid 551 and a threonine to alanine mutation at residue 552). The oxygen regulation of the polypeptide containing both the indicated internal deletion and the double point mutation is shown in the "mut" column, where a "+" indicates that the polypeptide is regulated, and is therefore unstable under non-hypoxic conditions.

FIG. 4 shows a model of regulated expression of HIF-1α. Putative regulatory sequences identified within the HIF-1α protein by deletion analysis are indicated. Potential interactions with regulatory proteins such as a phosphatase, kinase, or protease are also shown.

FIG. 5 is a bar graph illustrating the luciferase activity upon cotransfection of 293 cells with a reporter gene containing a hypoxic response element (that includes a HIF-1 binding site) with expression vector pCEP4 encoding (1) no protein; (2) full-length HIF-1α (amino acis 1–826); (3) HIF-1α (1–391/429–826, deletion only); (4) HIF-1αDP (deletion and a serine to glycine mutation at amino acid 551 and a threonine to alanine mutation at residue 552). Reporter gene expression is shown at 1% (black bars) and 20% $O_2$ (white bars).

FIG. 6 is a bar graph illustrating the luciferase activity upon cotransfection of Hep3B cells with a reporter gene containing a hypoxic response element (that includes a HIF-1 binding site) and with expression vector pCEP4 encoding (1) no protein; (2) HIF-1α; (3) HIF-1α (1–391/429–826, deletion only); (4) HIF-1αDP (deletion and a serine to glycine mutation at amino acid 551 and a threonine to alanine mutation at residue 552). Reporter gene expression is shown at 1% (black bars) and 20% $O_2$ (white bars).

## DETAILED DESCRIPTION OF THE INVENTION

It must be noted that as used herein and in the appended claims, the singular forms "a," "and," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the plasmid" includes reference to one or more plasmids and equivalents thereof known to those skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. Although any methods, devices and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods, devices and materials are now described.

All publications mentioned herein are incorporated herein by reference in full for the purpose of describing and disclosing the cell lines, vectors, and methodologies which are described in the publications which might be used in connection with the presently described invention. The publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

The invention provides a substantially pure stable hypoxia-inducible factor-1α (sHIF-1α) mutein. Wild-type, full-length HIF-1α is expressed at lower levels in nonhypoxic cells as compared to hypoxic cells (Wang, G. L., et al., *Proc. Natl. Acad. Sci. USA* 92:5510–5514, 1995; Wang, G. L., and Semenza, S. L., *J. Biol. Chem.* 270:1230–1237, 1995; Wang, G. L., and Semenza, S. L., *Curr. Opin. Hematol.* 3:156–162, 1992; Jiang, B. H., et al., *J. Biol. Chem.* 272:19253–19260, 1997, herein incorporated by reference) while sHIF-1α is stable under nonhypoxic as well as hypoxic conditions. Wild type HIF-1α and sHIF-1α are characterized as being able to form heterodimers with HIF-1β to form a DNA-binding protein, hypoxia inducible

factor-1 (HIF-1), a mammalian transcription factor. HIF-1 activates erythropoietin (EPO), vascular endothelial growth factor (VEGF), and glycolytic gene transcription.

## POLYNUCLEOTIDES AND POLYPEPTIDES

The term "mutein" as used herein refers to a variant form of HIF-1α polypeptide. HIF-1α polypeptide, upon dimerization with HIF-1β, is a DNA binding protein, which is characterized as activating structural gene expression where the promoter region of the structural gene contains a HIF-1 binding site (Semenza, G. L., et al., *Kid. Int.* 51:553–555, 1997; Iyer, N. V., et al., *Genes Dev.* 12:149–162, 1998, both herein incorporated by reference). Examples of such structural genes include erythropoietin (EPO), vascular endothelial growth hormone (VEGF) and glycolytic genes. HIF-1α migrates on SDS polyacrylamide gel electrophoresis with an apparent molecular mass of 120 kDa and has essentially the amino acid sequence as set forth in SEQ ID NO:1. The term HIF-1α includes the polypeptide as set forth in SEQ ID NO:1, and conservative variations of the polypeptide sequence. The term "conservative variant" as used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide. In a preferred embodiment, HIF-1α has the sequence as set forth in SEQ ID NO:1. HIF-1α is described in detail in copending application U.S. patent application Ser. No. 08/480,473, now U.S. Pat. No. 5,882,914 herein incorporated by reference.

In general, a mutein will have an amino acid sequence that differs from the native sequence by including substitutions, insertions, and/or deletions for example). Muteins are easily prepared using modem cloning techniques, or may be synthesized by solid state methods by site-directed mutagenesis. A mutein may include dominant negative forms of a polypeptide.

The invention provides a substantially pure stable hypoxia-inducible factor-1α (sHIF-1α) mutein. sHIF-1α polypeptide has a sequence as set forth in SEQ ID NO:1, wherein amino acids 392 to 428 are deleted therefrom, amino acid 551 is changed from a serine to any other amino acid, and amino acid 552 is changed from a threonine to any other amino acid. In one embodiment, amino acids 392 to 428 are deleted from SEQ ID NO:1 and amino acid 551 is changed from a serine to a glycine. In another embodiment, amino acids 392 to 428 are deleted from SEQ ID NO:1 and amino acid 552 is changed from a threonine to an alanine. In yet another embodiment, amino acids 392 to 428 are deleted from SEQ ID NO:1 and amino acid 551 is changed from a serine to a glycine and amino acid 552 is changed from a threonine to an alanine.

Without being bound by theory, two regions of full-length HIF-1α have been identified that are important for stable expression of HIF-1α. Region AB is located from about amino acid 392 to amino acid 552. Within this region, two sequences A and B, have been identified. In particular, sequence A is from amino acid 392 to amino acid 428 of SEQ ID NO:1, and sequence B is at about amino acid 429 to 522 of SEQ ID NO:1. Region C is located from about amino acid 703 to amino acid 726 of SEQ ID NO:1. A "mutation" in SEQ ID NO:1 refers to a deletion, insertion, mutation or substitution of one or more amino acids. Stable HIF-1α can be composed of a mutation or deletion in both regions A and B. Alternatively, stable HIF-1α can be composed of a deletion in region C. For example, regions A and B can be deleted, regions A and B can be mutated, or region A can be mutated and region B can be deleted, region A can be deleted and region B can be mutated, or region C can be mutated, or region C can be deleted. In one nonlimiting example, stable HIF-1α is composed of a deletion of amino acid 392 to amino acid 520 of SEQ ID NO:1. In another nonlimiting example, stable HIF-1α is composed of a deletion of amino acid 392 to 428 of SEQ ID NO:1, combined with point mutation of either amino acid 551 or 552, or combined with point mutation of both amino acid 551 and 552. The point mutation(s) can be combined with a deletion of amino acids 392 to amino acid 428 of SEQ ID NO:1, or the point mutation(s) can be combined with a deletion of amino acid 392 to any amino acid between amino acid 429 and amino acid 550, inclusive, of SEQ ID NO:1.

In yet another nonlimiting example, stable HIF-1α is composed of a deletion of amino acid 704 to amino acid 826 of SEQ ID NO:1. This deletion eliminates the transactivation domain (amino acid 786 to amino acid 826), and thus can result in a loss of biological activity. In one embodiment, stable HID-1α can be formed by deletion of amino acid 704 to amino acid 826 of SEQ ID NO:1, with the addition of a heterologous transactivation domain. The "heterologous" transactivation domain is a transactivation domain derived from a polypeptide other than HIF-1α. In one embodiment, the heterologous transactivation domain is a heterologous transactivation domain that is not affected by oxygen. In one nonlimiting example, the heterologous transactivation domain is a VP16 protein transactivation domain. In this embodiment, deletion of amino acid 391 to 704 is combined with a deletion of amino acid 704 to amino acid 826. A VP16 transactivation domain is then fused to amino acids 1 to amino acid 390 of the HIF-1α polypeptide. Additional combinations of the regions identified to be significant to the formation of sHIF-1α mutein will readily be apparent to one of skill in the art.

A "stable" HIF-1α is an HIF-1α polypeptide which has an increased half-life as compared to wild-type HIF-1α under nonhypoxic conditions. In one embodiment, in a given cell, sHIF-1α has the same half-life under hypoxic or nonhypoxic conditions. In another embodiment, a stable HIF-1α is present at the same concentration in cells exposed to nonhypoxic conditions as in cells exposed to hypoxic conditions.

The ability of wild-type HIF-1α to activate transcription is regulated by oxygen concentration independent of the effect of oxygen on HIF-1α protein stability (Jiang et al., 1997, supra). The region of sHIF-1α located from amino acid 576–785 is a negative regulatory domain that, when deleted, results in increased transcription under nonhypoxic conditions (Jiang et al., *J. Biol. Chem.* 272:19253, 1997, herein incorporated by reference). Thus, without being bound by theory, deletion of one or more amino acids in this sequence, such that the amino acid is replaced by a bond, results in a higher transcription rate, independent of the half life of the protein. Thus, deletion of amino acids 576–785 of HIF-1α can be combined with deletion of amino acids 392–428, and point mutation of amino acid 551 from a serine to a glycine, and point mutation of amino acid 552 from a threonine to an alanine, to yield a stable HIF-1α

polypeptide. Deletion of amino acid 576 to amino acid 785 of HIF-1α can also be combined with deletion of amino acids 392 to 520 to yield a stable HIF-1α polypeptide. Alternatively, deletion of amino acid 576 to amino acid 785 of HIF-1α can be combined to deletion of amino acid 704 to amino acid 826 (resulting in deletion of amino acid 576 to 826 of HIF-1α) to yield a stable HIF-1α polypeptide. Such combinations will readily be apparent to one of ordinary skill in the art.

The term "substantially pure" as used herein refers to HIF-1α which is substantially free of other proteins, lipids, carbohydrates or other materials with which it is naturally associated. One skilled in the art can purify HIF-1α using standard techniques for protein purification, such as DNA affinity chromatography (e.g., Wang, G. L., and Semenza, J., *J. Biol. Chem.* 270:1230–1237, 1995) and immunoprecipitation (e.g., Jiang, B. H., et al., *J. Biol. Chem.* 271:17771–17778, 1996). The substantially pure polypeptide will yield a single band on a nonreducing polyacrylamide gel. The purity of the HIF-1α polypeptide can also be determined by amino-terminal amino acid sequence analysis. HIF-1α protein includes functional fragments of the polypeptide, as long as the activity and the stability in nonhypoxic conditions of sHIF-1α remains. Smaller peptides containing the biological activity of sHIF-1α are thus included in the invention.

The invention provides polynucleotide sequences encoding sHIF-1α polypeptide having a sequence as set forth in SEQ ID NO:1, wherein amino acids 392 to 428 are deleted therefrom, amino acid 551 is changed from a serine to any other amino acid, and amino acid 552 is changed from a threonine to any other amino acid. These polynucleotides include DNA, cDNA, and RNA sequences which encode sHIF-1α. It is also understood that all polynucleotides encoding all or a portion of sHIF-1α are also included herein, as long as they encode a polypeptide with HIF-1α activity which is stable under hypoxic and nonhypoxic conditions. Such polynucleotides include naturally occurring, synthetic, and intentionally manipulated polynucleotides. For example, sHIF-1α polynucleotide may be subjected to site-directed mutagenesis. The polynucleotide sequence for sHIF-1α also includes antisense sequences. The polynucleotides of the invention include sequences that are degenerate as a result of the genetic code. There are 20 natural amino acids, most of which are specified by more than one codon. Therefore, all degenerate nucleotide sequences are included in the invention as long as the amino acid sequence of HIF-1α polypeptide is encoded by the nucleotide sequence is functionally unchanged.

Minor modifications of the sHIF-1α primary amino acid sequence may result in proteins which are stable under nonhypoxic conditions and have substantially equivalent activity as compared to the sHIF-1α polypeptide described herein. These minor modifications include the minor differences found in the sequence of HIF-1α polypeptide isolated from different species (e.g., human, mouse, and rat HIF-1α polypeptide). Such proteins include those as defined by the term "having essentially the amino acid sequence" of the sHIF-1α of the invention. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous, as those found in different species. All of the polypeptides produced by these modifications are included herein as long as the biological activity of sHIF-1α still exists, and the polypeptide is stable under nonhypoxic conditions as compared to wild-type HIF-1α. Further, deletions of one or more amino acids can also result in modification of the structure of the resultant molecule without significantly altering its biological activity. This can lead to the development of a smaller active molecule which would have broader utility. For example, one can remove amino or carboxy terminal amino acids which are not required for sHIF-1α biological activity.

Specifically disclosed herein is a DNA sequence encoding the human sHIF-1α mutein. The invention provides polynucleotide sequences encoding stable HIF-1α mutein having a sequence as set forth in SEQ ID NO:1, wherein amino acids 392 to 428 are deleted therefrom, amino acid 551 is changed from a serine to any other amino acid, and amino acid 552 is changed from a threonine to any other amino acid. The wild type HIF-1α contains an open reading frame encoding a polypeptide 826 amino acids in length. When amino acid 551 (serine) of SEQ ID NO:1 is replaced by another amino acid, such as an glycine, or amino acid 552 (threonine) of SEQ ID NO:1 is replaced by another amino acid, such as alanine, and one or more of amino acid 392 to amino acid 429 of SEQ ID NO:1 is replaced by a bond, the polynucleotide will encode a polypeptide that is decreased in length by a corresponding number of amino acids.

In another embodiment, the invention provides polynucleotides encoding sHIF-1α as well as nucleic acid sequences complementary to polynucleotides encoding sHIF-1α. The term "polynucleotide" or "nucleic acid sequence" refers to a polymeric form of nucleotides at least 10 bases in length. By "isolated polynucleotide" is meant a polynucleotide that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA) independent of other sequences. The nucleotides of the invention can be ribonucleotides, deoxyribonucleotides, or modified forms of either nucleotide. The term includes single and double stranded forms of DNA.

A complementary sequence may include an antisense nucleotide. When the sequence is RNA, the deoxynucleotides A, G, C, and T in the polynucleotide encoding sHIF-1α are replaced by ribonucleotides A, G, C, and U, respectively, Also included in the invention are fragments of the above-identified nucleic acid sequences that are at least bases in length, which is sufficient to permit the fragment to selectively hybridize to nucleic acid that encodes sHIF-1α, but not SEQ ID NO:1 under physiological conditions. Specifically, the fragments should selectively hybridize to nucleic acid encoding sHIF-1α polypeptide. The term "selectively hybridize" refers to hybridization under moderately or highly stringent conditions which excludes nonrelated nucleotide sequences.

In nucleic acid hybridization reactions, the conditions used to achieve a particular level of stringency' will vary, depending on the nature of the nucleic acids being hybridized. For example, the length, degree of complementarity, nucleotide sequence composition (e.g., GC v. AT content), and nucleic acid type (e.g., RNA v. DNA) of the hybridizing regions of the nucleic acids can be considered in selecting hybridization conditions. An additional consideration is whether one of the nucleic acids is immobilized, for example, on a filter.

An example of progressively higher stringency conditions is as follows: 2×SSC/0.1% SDS at about room temperature

(hybridization conditions); 0.2×SSC/0.1% SDS at about room temperature (low stringency conditions); 0.2×SSC/0.1% SDS at about 42° C. (moderate stringency conditions); and 0.1×SSC at about 68° C. (high stringency conditions). Washing can be carried out using only one of these conditions, e.g., high stringency conditions, or each of the conditions can be used, e.g., for 10–15 minutes each, in the order listed above, repeating any or all of the steps listed. However, as mentioned above, optimal conditions will vary, depending on the particular hybridization reaction involved, and can be determined empirically.

When using an sHIF-1α specific probe, it may be necessary to amplify the nucleic acid prior to binding with an sHIF-1α specific probe. Preferably, polymerase chain reaction (PCR) is used, however, other nucleic acid amplification procedures such as ligase chain reaction (LCR), ligated activated transcription (LAT) and nucleic acid sequence-based amplification (NASBA) may be used.

The sHIF-1α polynucleotide of the invention can be derived from a mammalian organism, and most preferably from human. Screening procedures which rely on nucleic acid hybridization make it possible to isolate any gene sequence from any organism, provided the appropriate probe is available. Oligonucleotide probes, which correspond to a part of the sequence encoding the protein in question, can be synthesized chemically. This requires that short, oligopeptide stretches of amino acid sequences must be known. The DNA sequence encoding the protein can be deduced from the genetic code, however, the degeneracy of the code must be taken into account. In a preferred embodiment, the probe can delineate between sHIF-1α and wild-type HIF-1α.

It is possible to perform a mixed addition reaction when the sequence is degenerate. This includes a heterogeneous mixture of denatured double-stranded DNA. For such screening, hybridization is preferably performed on either single-stranded DNA or denatured double-stranded DNA. Hybridization is particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. In other words, by using stringent hybridization conditions directed to avoid nonspecific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Sambrook et al., *Molecular Cloning: A Laboratory Manual* 2nd Ed.; Cold Spring Harbor Laboratory Press, Plainview, N.Y., 1998).

The development of specific DNA sequences encoding sHIF-1α can also be obtained by site-directed mutagenesis of a nucleic acid sequence encoding SEQ ID NO:1 or chemical manufacture of a DNA sequence to provide the necessary codons for the polypeptide of interest. The synthesis of DNA sequences is frequently the method of choice when the entire sequence of amino acid residues of the desired polypeptide product is known.

A cDNA expression library, such as in phage lambda gt11, can be screened indirectly for sHIF-1α peptides having at least one epitope, using antibodies specific for sHIF-1α. Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of sHIF-1α cDNA.

DNA sequences encoding sHIF-1α can be expressed in vitro by DNA transfer into a suitable host cell. "Host cells" are cells in which a vector can be propagated and its DNA expressed. Host cells include both prokaryotic and eukaryotic cells. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication. However, such progeny are included when the term "host cell" is used. Methods of stable transfer, meaning that the foreign DNA is continuously maintained in the host, are known in the art.

"Modified" versions of the specific sHIF-1α can be engineered to further enhance stability, biological acitvity, production, purification, or yield of the expressed product. For example, the expression of a fusion protein or a cleavable fusion protein comprising the sHIF-1α and a heterologous protein can be engineered. Such a fusion protein can be readily isolated by affinity chromatography, e.g., by immobilization on a column specific for the heterologous protein. Where a cleavage site is engineered between the HIF-1α moiety and the heterologous protein, the HIF-1α polypeptide can be released from the chromatographic column by treatment with an appropriate enzyme or agent that digests at the cleavage site (Booth et al., *Immunol. Lett.* 19:65–708, 1988; Gardella et al., *J. Biol. Chem.* 265:15854–15859, 1990).

In the present invention, the sHIF-1α polynucleotide sequences may be inserted into an expression vector. The term "expression vector" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of the sHIF-1α genetic sequences. Polynucleotide sequence which encode sHIF-1α can be operatively linked to expression control sequences. "Operatively linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. An expression control sequence operatively linked to a coding sequence is ligated such that expression of the coding sequence is achieved under conditions compatible with the expression control sequences. As used herein, the term "expression control sequences" refers to nucleic acid sequences that regulate the expression of a nucleic acid sequence to which it is operatively linked. Expression control sequences are operatively linked to a nucleic acid sequence when the expression control sequences control and regulate the transcription and, as appropriate, translation of the nucleic acid sequence. Thus expression control sequences can include appropriate promoters, enhancers, transcription terminators, as start codon (i.e., ATG) in front of a protein-encoding gene, splicing signal for introns, maintenance of the correct reading frame of that gene to permit proper translation of mRNA, and stop codons. The term "control sequences" is intended to included, at a minimum, components whose presence can influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences. Expression control sequences can include a promoter.

By "promoter" is meant minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell-type specific, tissue-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the gene. Both constitutive and inducible promoters, are included in the invention (see e.g., Bitter et al., *Methods in Enzymology* 153:516–544, 1987). For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage γ, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used. When cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (e.g., metallothionein or elongation factor-1α

promoter) or from mammalian viruses (e.g., the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter; the cytomegalovirus promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the nucleic acid sequences of the invention.

In the present invention, the polynucleotide encoding sHIF-1α may be inserted into an expression vector which contains a promoter sequence which facilitates the efficient transcription of the inserted genetic sequence of the host. The expression vector typically contains an origin of replication, a promoter, as well as specific genes which allow phenotypic selection of the transformed cells. Vectors suitable for use in the present invention include, but are not limited to the T7-based expression vector for expression in bacteria (Rosenberg et al., *Gene* 56:125, 1987), the pMSXND expression vector for expression in mammalian cells (Lee and Nathans, *J. Biol. Chem.* 263:3521, 1988) and baculovirus-derived vectors for expression in insect cells. The DNA segment can be present in the vector operably linked to regulatory elements, for example, a promoter (e.g., T7, metallothionein I, or polyhedrin promoters).

In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review see, *Current Protocols in Molecular Biology,* Vol. 2, Ch. 3, Ed. Ausubel et al., Greene Publish. Assoc. & Wiley Interscience, 1988; Grant et al., "Expression and Secretion Vectors for Yeast," in *Methods in Enzymology,* Vol. 153, pp. 516–544, Eds. Wu & Grossman, 31987, Acad. Press, N.Y., 1987; Glover, *DNA Cloning,* Vol. II, Ch. 3, IRL Press, Wash., D.C., 1986; and Bitter, "Heterologous Gene Expression in Yeast," *Methods in Enzymology,* Vol. 152, pp. 673–684, Eds. Berger & Kimmel, Acad. Press, N.Y., 1987; and *The Molecular Biology of the Yeast Saccharomyces,* Vols. I and II, Eds. Strathern et al., Cold Spring Harbor Press, 1982. A constitutive yeast promoter such as ADH or LEU2 or an inducible promoter such as GAL may be used ("Cloning in Yeast," R. Rothstein In: *DNA Cloning, A Practical Approach,* Vol. 11, Ch. 3, Ed. D. M. Glover, IRL Press, Wash., D.C., 1986). Alternatively, vectors may be used which promote integration of foreign nucleic acid sequences into the yeast chromosome.

Mammalian expression systems which utilize recombinant viruses or viral elements to direct expression may be engineered. For example, when using adenovirus expression vectors, the sHIF-1α coding sequence may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. Alternatively, the vaccinia virus 7.5K promoter may be used. (e.g., see, Mackett et al., *Proc. Natl. Acad. Sci. USA* 79:7415–7419, 1982; Mackett et al., *J. Virol.* 49:857–864, 1984; Panicali et al., Proc. Natl. Acad. Sci USA 79:4927–4931, 1982). Of particular interest are vectors based on bovine papilloma virus which have the ability to replicate as extrachromosomal elements (Sarver, et al., *Mol. Cell. Biol.* 1:486, 1981). Shortly after entry of this nucleic acid into mouse cells, the plasmid replicates to about 100 to 200 copies per cell. Transcription of the inserted cDNA does not require integration of the plasmid into the host's chromosome, thereby yielding a high level of expression. These vectors can be used for stable expression by including a selectable marker in the plasmid, such as, for example, the neo gene. Alternatively, the retroviral genome can be modified for use as a vector capable of introducing and directing the expression of the sHIF-1α gene in host cells (Cone & Mulligan, *Proc. Natl. Acad. Sci. USA* 81:6349–6353, 1984). High level expression may also be achieved using inducible promoters, including, but not limited to, the metallothionein IIA promoter and heat shock promoters.

Polynucleotide sequences encoding sHIF-1α can be expressed in either prokaryotes or eukaryotes. Hosts can include microbial, yeast, insect and mammalian organisms. Methods of expressing DNA sequences having eukaryotic or viral sequences in prokaryotes are well known in the art. Biologically functional viral and plasmid DNA vectors capable of expression and replication in a host are known in the art. Such vectors are used to incorporate DNA sequences of the invention.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with sHIF-1α cDNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. For example, following the introduction of foreign nucleic acid, engineered cells may be allowed to grow for 1–2 days in an enriched media, and then are switched to a selective media. A number of selection systems may be used, including, but not limited to the herpes simplex virus thymidine kinase gene (Wigler, et al., *Cell* 11:223, 1977), hypoxanthine-guanine phosphoribosyltransferase gene (Szybalska & Szybalski, *Proc. Natl. Acad. Sci. USA* 48:2026, 1962), and the adenine phosphoribosyltransferase (Lowy, et al., *Cell* 22:817, 1980) genes can be employed in tk–, hgprt$^-$ or aprt$^-$ cells respectively. Additionally, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., *Natl. Acad. Sci. USA* 77:3567, 1980; O'Hare, et al., *Proc. Natl. Acad. Sci. USA* 78:1527, 1981); the gpt gene, which confers resistance to mycophenolic acid (Mulligan & Berg, *Proc. Natl. Acad. Sci. USA* 78:2072, 1981; the neo gene, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., *J. Mol Biol.* 150:1, 1981); and the hygro gene, which confers resistance to hygromycin (Santerre, et al., *Gene* 30:147, 1984) genes. Recently, additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, *Proc. Natl. Acad. Sci. USA* 85:8047, 1988); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., In: *Current Communications in Molecular Biology,* Cold Spring Harbor Laboratory ed., 1987).

By "transformation" is meant a genetic change induced in a cell following incorporation of new DNA (i.e., DNA exogenous to the cell). Where the cell is a mammalian cell, the genetic change is generally achieved by introduction of the DNA into the genome of the cell (i.e., stable).

By "transformed cell" is meant a cell into which (or into an ancestor of which has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding sHIF-1α. Transformation of a host cell with recombinant DNA may be carried out by conventional techniques as are well known to those skilled in the art. Where the host is prokaryotic, such as *E. coli,* competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the $CaCl_2$ method using procedures well known in the art.

Alternatively, $MgCl_2$ or RbCl can be used. Transformation can also be performed after forming a protoplast of the host cell if desired.

When the host is a eukaryote, such methods of transfection of DNA as calcium phosphate co-precipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors may be used. Eukaryotic cells can also be cotransformed with DNA sequences encoding the sHIF-1α of the invention, and a second foreign DNA molecule encoding a selectable phenotype, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40), adenovirus, or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein (see for example, *Eukaryotic Viral Vectors,* Cold Spring Harbor Laboratory, Gluzman, ed., 1982).

Isolation and purification of microbial expressed polypeptide, or fragments thereof, provided by the invention, may be carried out by conventional means including preparative chromatography and immunological separations involving monoclonal or polyclonal antibodies.

## ANTIBODIES

The HIF-1α polypeptides of the invention can also be used to produce antibodies which are immunoreactive or selectively bind to epitopes of the sHIF-1α polypeptides. An antibody which "selectively binds" to sHIF-1α is an antibody that binds sHIF-1α with a higher affinity the antibody binds to wild-type HIF-1α. Thus, antibodies of the invention can be used to distinguish the presence of sHIF-1α mutein from wild-type HIF-1α polypeptide. Antibody which consists essentially of pooled monoclonal antibodies with different epitopic specificities, as well as distinct monoclonal antibody preparations are provided. Monoclonal antibodies are made from antigen containing fragments of the protein by methods well known in the art (Kohler et al. *Nature* 256:495, 1975; *Current Protocols in Molecular Biology,* Ausubel et al., ed., 1989).

The term "antibody" as used in this invention includes intact molecules as well as fragments thereof, such as Fab, F(ab')2, and Fv which are capable of binding the epitopic determinant. These antibody fragments retain some ability to selectively bind with its antigen or receptor and are defined as follows:

(1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain;

(2) Fab', the fragment of an antibody molecule can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule;

(3) $(Fab')_2$, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; $F(ab')_2$ is a dimer of two Fab' fragments held together by two disulfide bonds;

(4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and

(5) Single chain antibody ("SCA"), defined as a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

Methods of making these fragments are known in the art. See, for example, Harlow and Lane, *Antibodies: A Laboratory Manual,* Cold Spring Harbor Laboratory, New York, 1988, incorporated herein by reference.

As used in this invention, the term "epitope" means any antigenic determinant on an antigen to which the paratope of an antibody binds. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

Antibodies which selectively bind to the sHIF-1α polypeptide of the invention, can be prepared using an intact polypeptide or fragments containing small peptides of interest as the immunizing antigen. The polypeptide or a peptide used to immunize an animal can be derived from translated cDNA or chemical synthesis which can be conjugated to a carrier protein, if desired. Such commonly used carriers which are chemically coupled to the peptide include keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), and tetanus toxoid. The coupled peptide is then used to immunize the animal (e.g., a mouse, a rat, or a rabbit).

If desired, polyclonal or monoclonal antibodies can be further purified, for example, by binding to and elution from a matrix to which the polypeptide or a peptide to which the antibodies were raised is bound. Those of skill in the art will know of various techniques common in the immunology arts for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies. See, for example, Coligan et al., Unit 9, *Current Protocols in Immunology,* Wiley Interscience, 1994, herein specifically incorporated by reference.

It is also possible to use the anti-idiotype technology to produce monoclonal antibodies which mimic an epitope. For example, an anti-idiotypic monoclonal antibody made to a first monoclonal antibody will have a binding domain in the hypervariable region which is the "image" of the epitope bound by the first monoclonal antibody.

For purposes of the invention, an antibody or nucleic acid probe specific for sHIF-1α may be used to detect sHIF-1α polypeptide or polynucleotide in biological fluids, cultured cells or tissues. The antibody reactive with sHIF-1α or the nucleic acid probe is preferably labeled with a compound which allows detection of binding to sHIF-1α. Any specimen containing a detectable amount of antigen or polynucleotide can be used.

## THERAPEUTIC INTERVENTION

The invention provides methods for treatment of HIF-1-mediated disorders, including hypoxia-mediated tissue damage, which are improved or ameliorated by modulation of HIF-1 gene expression or activity. The term "modulate" envisions the induction or augmentation of HIF-1 expression when appropriate. The term "ameliorate" denotes a lessening of the detrimental effect of the associated disease in the subject receiving therapy. Where expression or augmentation of expression of HIF-1 is desirable, the method of the treatment includes administration of substantially purified sHIF-1α polypeptide or polynucleotide.

According to the method of the invention, substantially purified sHIF-1α mutein or the polynucleotide sequence encoding sHIF-1α is introduced into a human patient for the treatment or prevention of HIF-1-mediated disorders. The

appropriate human patient is a subject suffering from a HIF-1-mediated disorder, such as an ischemic disease, or a hypoxia-related disorder (for example, coronary, cerebral, or peripheral arterial disease).

The present invention provides the introduction of polynucleotides encoding sHIF-1α for the treatment of hypoxia-related disorders, which are improved or ameliorated by expression of the HIF-1α polypeptide. Such therapy would achieve its therapeutic effect by introduction of the sHIF-1α polynucleotide into cells exposed to hypoxic conditions. HIF-1α is thus expressed in both the hypoxic and surrounding nonhypoxic tissues, such that it can dimerize with HIF-1β (which is present in excess in hypoxic and nonhypoxic cells), and activate the transcription of downstream target genes. Examples of genes which can be activated by HIF-1 are vascular endothelial growth factor, glucose transporters, and glycolytic enzymes. These genes mediate important adaptive responses to hypoxia including angiogenesis and glycolysis.

Delivery of sHIF-1α polynucleotide can be achieved using a recombinant expression vector such as a chimeric virus or a colloidal dispersion system. Especially preferred for therapeutic delivery of sequences is the use of targeted liposomes.

Various viral vectors which can be utilized for gene therapy as taught herein include adenovirus, adeno-associated virus, herpes virus, vaccinia, or, preferably, an RNA virus such as a retrovirus. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). Preferably, when the subject is a human, a vector such as the gibbon ape leukemia virus (GaLV) is utilized. A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated. By inserting a sHIF-1α sequence of interest into the viral vector, along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target specific. Retroviral vectors can be made target specific by attaching, for example, a sugar, a glycolipid, or a protein. Preferred targeting is accomplished by using an antibody to target the retroviral vector. Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific polynucleotide sequences which can be inserted into the retroviral genome or attached to a viral envelope to allow target specific delivery of the retroviral vector containing the sHIF-1α polynucleotide.

Since recombinant retroviruses are defective, they require assistance in order to produce infectious vector particles. This assistance can be provided, for example, by using helper cell lines that contain plasmids encoding all of the structural genes of the retrovirus under the control of regulatory sequences within the LTR. These plasmids are missing a nucleotide sequence which enables the packaging mechanism to recognize an RNA transcript for encapsidation. Helper cell lines which have deletions of the packaging signal include, but are not limited to Ψ2, PA317 and PA12, for example. These cell lines produce empty virions, since no genome is packaged. If a retroviral vector is introduced into such cells in which the packaging signal is intact, but the structural genes are replaced by other genes of interest, the vector can be packaged and vector virion produced.

Alternatively, NIH 3T3 or other tissue culture cells can be directly transfected with plasmids encoding the retroviral structural genes gag, pol and env, by conventional calcium phosphate transfection. These cells are then transfected with the vector plasmid containing the genes of interest. The resulting cells release the retroviral vector into the culture medium.

Another targeted delivery system for HIF-1 polynucleotides is a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome. Liposomes are artificial membrane vesicles which are useful as delivery vehicles in vitro and in vivo. It has been shown that large unilamellar vesicles (LW), which range in size from 0.2–4.0 μm can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley et al., *Trends Biochem. Sci.* 6:77, 1981). In addition to mammalian cells, liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells. In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the genes of interest at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino et al. *Biotechniques* 6:682, 1988).

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with sterols, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidyl-glycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides. Particularly useful are d-iacylphosphatidylglycerols, where the lipid moiety contains from 14–18 carbon atoms, particularly from 16–18 carbon atoms, and is saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

The targeting of liposomes can be classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated

into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association with the liposomal bilayer. Various linking groups can be used for joining the lipid chains to the targeting ligand.

sHIF-1α polypeptide can be used in therapeutic administration. For such administration the polypeptide must be sterile. Sterility is readily accomplished by sterile filtration through (e.g., 0.2 micron) membranes. The compound of the invention ordinarily will be stored as unit or multidose containers, for example, sealed ampules or vials, as an aqueous solution, as it is highly stable to thermal and oxidative denaturation. Lyophilized formulations for reconstitution are also acceptable. The polypeptide will be administered as a pharmaceutical composition (see below).

The invention also describes a method of treating a subject having a hypoxia related disorder by administering to the subject a therapeutically-effective amount of cells expressing sHIF-1α. "Therapeutically-effective" as used herein, refers to that amount of cells that is of sufficient quantity to alleviate a symptom of the disease or to ameliorate the hypoxia-related disorder. Transduction of the cell is performed in vitro, generally with isolated cell populations or cell lines. The cells may be xenogeneic, allogeneic, syngeneic or autologous, preferably autologous, in order to reduce adverse immune responses. The cells may be administered in any physiologically acceptable medium, normally intravascularly, although they may also be introduced into tissue surrounding a vessel or other convenient site, where the cells may find an appropriate site for expansion and differentiation. "Ameliorate" refers to lessening or lowering the disease's or disorder's detrimental effect in the patient receiving the therapy.

Any of the transplantation or implantation procedures known in the art can be utilized. For example, the selected cells or cells of interest can be surgically implanted into the recipient or subject. Transplantation or implantation is typically by simple injection through a hypodermic needle having a bore diameter sufficient to permit passage of a suspension of cells therethrough without damaging the cells or tissue coating. For implantation, the typically encapsulated or coated cells are formulated as pharmaceutical compositions together with a pharmaceutically-acceptable carrier. Such compositions contain a sufficient number of coated transplant cells which can be injected into, or administered through a laparoscope to, a subject. Usually, at least about $1\times10^4$ to $1\times10^5$ cells will be administered, preferably $1\times10^6$ or more. The cells may be frozen at liquid nitrogen temperatures and stored for long periods of time, being capable of use on thawing. Once thawed, the cells may be expanded. Further, the cells can be administered in an encapsulated form or non-encapsulated form. Preferably the cells are encapsulated.

While not required, it may be desirable to administer an immunosuppressive agent to a recipient of the cells, prior to, simultaneous with, and/or after transplantation. In particular, an immunosuppressive agent can be utilized with xenogeneic or allogeneic cells expressing sHIF-1α. An agent such as Cyclosporine A (CsA) is preferable, however other immune suppressive agents can be used, such as rapamycin, desoxyspergualine, FK506 and like. These agents are administered to cause an immunosuppressive effect in the subject, such that the transplanted cells are not rejected by that subject's immune system. Typically, the immunosuppressive agent is administered continuously through-out the transplant treatment typically over a period of days or weeks; for example, CsA treatment ranges from about 2 to about 20 days at a dosage range of about 5 to 40 mg per kilogram of body weight per day. The agent can be administered by a variety of means, including parenteral, subcutaneous, intrapulmonary, oral, intranasal administration and the like. Preferably, dosing is given by oral administration.

The cells expressing HIF-1α also can be encapsulated prior to transplantation. Although the cells are typically microencapsulated, they can be encased in various types of hollow fibers or in a block of encapsulating material. A variety of microencapsulation methods and compositions are known in the art. A number of microencapsulation methods for use in transplant therapy have focused on the use of alginate polymers or agarose to supply the encapsulation compositions. Alginates are linear polymers of mannuronic and guluronic acid residues which are arranged in blocks of several adjacent guluronic acid residues forming guluronate blocks and block of adjacent mannuronic acid residues forming mannuronate blocks, interspersed with mixed, or heterogenous blocks of alternating guluronic and mannuronic acid residues. Generally, monovalent cation alginate salts are soluble, e.g., Na-alginate.

Divalent cations, such as $Ca^{++}$, $Ba^{++}$ or $Sr^{++}$, tend to interact with guluronate, and the cooperative binding of these cations within the guluronate blocks provides the primary intramolecular crosslinking responsible for formation of stable ion-paired alginate gels. Alginate encapsulation methods generally take advantage of the gelling of alginate in the presence of these divalent cation solutions. In particular, these methods involve the suspension of the material to be encapsulated, in a solution of monovalent cation alginate salt, e.g., sodium. Droplets of the solution are then generated in air and collected in a solution of divalent cations, e.g., $CaCl_2$. The divalent cations interact with the alginate at the phase transition between the droplet and the divalent cation solution resulting in the formation of a stable alginate gel matrix being formed. Generation of alginate droplets has previously been carried out by a number of methods. For example, droplets have been generated by extrusion of alginate through a tube by gravitational flow, into a solution of divalent cations. Similarly, electrostatic droplet generators which rely on the generation of an electrostatic differential between the alginate solution and the divalent cation solution have been described. The electrostatic differential results in the alginate solution being drawn through a tube, into the solution of divalent cations. Methods have been described wherein droplets are generated from a stream of the alginate solution using a laminar air flow extrusion device. Specifically, this device comprises a capillary tube within an outer sleeve. Air is driven through the outer sleeve and the polymer solution is flow-regulated through the inner tube. The air flow from the outer sleeve breaks up the fluid flowing from the capillary tube into small droplets (see U.S. Pat. No. 5,286,495). For a general discussion of droplet generation in encapsulation processes, see, e.g., M. F. A. Goosen, Fundamentals of Animal Cell Encapsulation and Mobilization, Ch. 6, pp. 114–142 (CRC Press, 1993).

Attempts to transplant organ tissues into genetically dissimilar hosts without immunosuppression are generally defeated by the immune system of the host. Accordingly, attempts have been made to provide other effective protective barrier coatings, e.g., by microencapsulation, to isolate the transplant tissues from the host immune system. Successful cell or tissue transplants generally require a coating that will prevent their destruction by a host's immune system, prevent fibrosis, and will be permeable to and allow a free diffusion of the nutrients to the coated transplant and

removal of the secretory and waste products from the coated transplant. Viable tissue and cells have been successfully immobilized in alginate capsules coated with polylysine (see above and *J. Pharm. Sci.* 70:351–354, 1981). The development of transplants encapsulated in calcium alginate capsules reacted with polylysine is also described, for example, in U.S. Pat. Nos. 4,673,566, 4,689,293, 4,789,550, 4,806,355, and 4,789,550. U.S. Pat. No. 4,744,933 describes encapsulating solutions containing biologically active materials in a membrane of inter-reacted alginate and polyamino acid. U.S. Pat. No. 4,696,286 reports a method for coating transplants suitable for transplantation into genetically dissimilar individuals. The method involves coating the transplant with a surface conforming bonding bridge of a multifunctional material that binds chemically to a surface component of the transplant, which is enveloped in a semipermeable, biologically compatible layer of a polymer that binds chemically to the bonding bridge layer. A method for introducing a second alginate gel coating to cells already coated with polylysine alginate is described in U.S. Pat. No. 5,227,298. Both the first and second coating of this method require stabilization by polylysine.

Encapsulation methods applied to make these materials have comprised a procedure for forming droplets of the encapsulating medium and the biological material and a procedure for solidifying the encapsulating medium. Agarose encapsulated materials have been formed by chilling an emulsion of agarose droplets containing biological materials as shown by Nilsson, et al., *Nature* 302:629–630 (1983) and Nilsson, et al., *Eur. J Appl. Microbiol. B-iotechnol.* 17:319–326 (1983). Injection of droplets of polymer containing biological materials into a body of coolant such as concurrently liquid stream has been reported by Gin, et al., *J. Microencapsulation* 4:329–242 (1987).

## PHARMACEUTICAL COMPOSITIONS

This invention involves administering to a subject a therapeutically effective dose of a pharmaceutical composition containing the compounds of the present invention and a pharmaceutically acceptable carrier. "Administering" the pharmaceutical composition of the present invention may be accomplished by any means known to the skilled artisan.

The pharmaceutical compositions are preferably prepared and administered in dose units. Solid dose units are tablets, capsules and suppositories. For treatment of a patient, depending on activity of the compound, manner of administration, nature and severity of the disorder, age and body weight of the patient, different daily doses are necessary. Under certain circumstances, however, higher or lower daily doses may be appropriate. The administration of the daily dose can be carried out both by single administration in the form of an individual dose unit or else several smaller dose units and also by multiple administration of subdivided doses at specific intervals.

The pharmaceutical compositions according to the invention are in general administered topically, orally, intravenously, or by another parenteral route, or as implants, or even rectal use is possible in principle. Suitable solid or liquid pharmaceutical preparation forms are, for example, granules, powders, tablets, coated tablets, (micro)capsules, suppositories, syrups, emulsions, suspensions, creams, aerosols, drops or injectable solution in ampule form and also preparations with protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as disintegrants, binders, coating agents, swelling agents, lubricants, flavorings, sweeteners or solubilizers are customarily used as described above. The pharmaceutical compositions are suitable for use in a variety of drug delivery systems. For a brief review of present methods for drug delivery, see Langer, *Science,* 249:1527–1533, 1990, which is incorporated herein by reference.

For delivery of sHIF-1α mutein, the formulations are prepared by contacting sHIF-1α mutein uniformly and intimately with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product into the desired formulation. Preferably the carrier is a parenteral carrier, more preferably a solution that is isotonic with the blood of the recipient. Examples of such carrier vehicles include water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils and ethyl oleate are also useful herein, as well as liposomes. Generally, the carrier can contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, e.g., buffers and preservatives, as well as low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose or dextrans, chelating agents such as EDTA, or other excipients.

The composition herein is also suitably administered by sustained release systems. Suitable examples of sustained release compositions include semipermeable polymer matrices in the form of shaped articles, e.g., films, microcapsules, or microspheres. Sustained release matrices include, for example, polyactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ-ethyl-L-glutamate (Sidman et al., *Biopolymers* 22:547–556, 1983), or poly-D-(−)-3-hydroxybutyric acid (EP 133,988). Sustained release compositions also include one or more liposomally entrapped compounds of formula I. Such compositions are prepared by methods known per se, e.g., as taught by Epstein et al. *Proc. Natl. Acad. Sci. USA* 82:3688–3692, 1985. Ordinarily, the liposomes are of the small (200–800 Å) unilamellar type in which the lipid content is greater than about 30 mol % cholesterol, the selected proportion being adjusted for the optimal therapy.

The pharmaceutical compositions according to the invention may be administered locally or systemically. By "therapeutically effective dose" is meant the quantity of a compound according to the invention necessary to prevent, to cure or at least partially arrest the symptoms of the disorder and its complications. Amounts effective for this use will, of course, depend on the severity of the disease and the weight and general state of the patient. Typically, dosages used in vitro may provide useful guidance in the amounts useful for in situ administration of the pharmaceutical composition, and animal models may be used to determine effective dosages for treatment of particular disorders. Various considerations are described, e.g., in Gilman et al., eds., *Goodman And Gilman's: The Pharmacological Bases of Therapeutics,* 8th ed., Pergamon Press, 1990; and *Remington's Pharmaceutical Sciences,* 17th ed., Mack Publishing Co., Easton, Pa., 1990; each of which is herein incorporated by reference.

The following examples are intended to illustrate but not limit the invention. While they are typical of those that might be used, other procedures known to those skilled in the art may alternatively be used.

## EXAMPLE 1

### Generation of a Constitutively Expressed Form of HIF-1α

It has previously been shown (Jiang et al., *J. Biol. Chem* 272:19253, 1997; Pugh et al., *J. Biol. Chem.* 272:11205) that

a fusion protein consisting of the GAL4 DNA binding domain fused to HIF-1α residues 531–826 is a constitutively expressed protein that can activate transcription of reporter genes containing GAL4 binding sites. However, these GAL4/HIF-1α constructs do not activate the normal target genes regulated by HIF-1. To generate a constitutively expressed form of HIF-1α, two series of deletion constructs were produced, one in which the deletions began at the carboxyl-terminal end of the molecule (amino acid 826) and extended towards the amino terminus, and one in which the deletions began at amino acid 392 and extended towards the carboxyl terminus.

Each of these constructs was expressed in mammalian cells under nonhypoxic (20% $O_2$) or hypoxic (1% $O_2$) conditions, and the expression of endogenous full length HIF-1α and transfected deleted HIF-1α was quantitated by immunoblot assay using affinity-purified anti-HIF-1α antibodies. These studies revealed that endogenous HIF-1α showed regulated expression (more protein expressed in cells at 1% $O_2$ than in cells at 20% $O_2$). In addition the studies showed that C-terminal deletion to amino acid 726 had no effect on the regulation of HIF-1α protein expression by $O_2$ concentration, whereas deletion to amino acid 703 or beyond resulted in loss of regulation (i.e., constitutive expression, see FIG. 2). Internal deletions extending from amino acid 392 through 517 had no effect on expression, whereas deletion of amino acid 392 to amino acid 521 resulted in loss of regulation (see FIG. 3). In addition, the missense mutations S551G/T552A (a serine to glycine and threonine to alanine substitution at amino acid 551 and 552, respectively) resulted in loss of regulation of the internal deletion constructs that otherwise showed regulation (i.e., deletions extending from amino acid 392 to anywhere between amino acid 429 and 517). These missense mutations alone did not cause dysregulated expression of full-length HIF-1α (amino acids 1–826, see FIG. 3).

The results suggested that there were two regions of HIF-1α that were required for regulated expression, such that deletion of either region resulted in dysregulated expression (see FIG. 4). The first of these regions is region AB (amino acid 392–552). Within this internal region, two sequences (A and B) were identified that appeared functionally redundant, since the presence of either sequence was sufficient for regulation. One of these sequences (A) was identified by the 392–428 deletion and the other sequence (B) was identified by the 392–520 deletion, or the S551G/T552A point mutations. This latter result suggested that the serine and/or threonine residue was subjected to phosphorylation/dephosphorylation which could be disrupted by the 392–520 deletion. Since loss of the serine/threonine sequence mimicked hypoxia, these results suggest phosphorylation of serine 551 and/or threonine 552 under nonhypoxic conditions and dephosphorylation under hypoxic conditions. Based upon the redundancy of A and B, it is possible that a phosphatase may also bind at the A site and dephosphorylate a nearby serine or threonine reside.

Region C is defined by the different effects of deletions encompassing amino acids 704 to 826 as compared to deletions encompassing amino acids 727 to 826. Loss of region C is not redundant with the loss of region AB, thus it is likely that this region will be involved in some other function related to regulation of HIF-1α stability. Without being bound by theory, it is possible this region is involved in ubiquitination or proteolysis.

A powerful transactivation domain is located between amino acids 786 and 826. As a result, although HIF-1α (amino acid 1–703) is constitutively expressed, it is not as biologically active as full-length HIF-1α. In order to determine if sHIF-1α would demonstrate increased biological activity compared to full-length HIF-1α cotransfection experiments using the deletion/point mutant HIF-1α (1–391/512–826/S551G/T552A), a stable HIF-1α, were performed. Either 293 cells (see FIG. 5) or Hep3B cells (see FIG. 6) were cotransfected with a reporter gene containing a hypoxia response element that includes an HIF-1 binding site, and with mammalian expression vector pCEP4 (Invitrogen) encoding either (1) no protein, (2) HIF-1α (1–826), (3) HIF-1α (1–391/429–826) (deletion only), or (4) stable HIF-1α (HIF-1αDP, a form of sHIF-1α which contains 1–391/512–826/S551G/T552A). Endogenous HIF-1β is constitutively expressed in these cells at levels in excess of HIF-α expression. In both cell types, HIF-1αDP (sHIF-1α) mediated significantly greater reporter gene expression in cells exposed to 20% $O_2$, due to the presence of higher levels of biologically active HIF-1α (note that HIF-1α is normally expressed only at 1% $O_2$). These results demonstrate a constitutively-expressed and biologically active form of HIF-1α has been generated.

Although the invention has been described with reference to the presently preferred embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 2

<210> SEQ ID NO 1
<211> LENGTH: 3736
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
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<400> SEQUENCE: 1

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                                  1               5
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gac aag aaa aag ata agt tct gaa cgt cga aaa gaa aag tct cga gat      100
Asp Lys Lys Lys Ile Ser Ser Glu Arg Arg Lys Glu Lys Ser Arg Asp
     10                  15                  20

gca gcc aga tct cgg cga agt aaa gaa tct gaa gtt ttt tat gag ctt      148
Ala Ala Arg Ser Arg Arg Ser Lys Glu Ser Glu Val Phe Tyr Glu Leu
 25                  30                  35                  40

gct cat cag ttg cca ctt cca cat aat gtg agt tcg cat ctt gat aag      196
Ala His Gln Leu Pro Leu Pro His Asn Val Ser Ser His Leu Asp Lys
                 45                  50                  55

gcc tct gtg atg agg ctt acc atc agc tat ttg cgt gtg agg aaa ctt      244
Ala Ser Val Met Arg Leu Thr Ile Ser Tyr Leu Arg Val Arg Lys Leu
             60                  65                  70

ctg gat gct ggt gat ttg gat att gaa gat gac atg aaa gca cag atg      292
Leu Asp Ala Gly Asp Leu Asp Ile Glu Asp Asp Met Lys Ala Gln Met
         75                  80                  85

aat tgc ttt tat ttg aaa gcc ttg gat ggt ttt gtt atg gtt ctc aca      340
Asn Cys Phe Tyr Leu Lys Ala Leu Asp Gly Phe Val Met Val Leu Thr
     90                  95                 100

gat gat ggt gac atg att tac att tct gat aat gtg aac aaa tac atg      388
Asp Asp Gly Asp Met Ile Tyr Ile Ser Asp Asn Val Asn Lys Tyr Met
105                 110                 115                 120

gga tta act cag ttt gaa cta act gga cac agt gtg ttt gat ttt act      436
Gly Leu Thr Gln Phe Glu Leu Thr Gly His Ser Val Phe Asp Phe Thr
                125                 130                 135

cat cca tgt gac cat gag gaa atg aga gaa atg ctt aca cac aga aat      484
His Pro Cys Asp His Glu Glu Met Arg Glu Met Leu Thr His Arg Asn
            140                 145                 150

ggc ctt gtg aaa aag ggt aaa gaa caa aac aca cag cga agc ttt ttt      532
Gly Leu Val Lys Lys Gly Lys Glu Gln Asn Thr Gln Arg Ser Phe Phe
        155                 160                 165

ctc aga atg aag tgt acc cta act agc cga gga aga act atg aac ata      580
Leu Arg Met Lys Cys Thr Leu Thr Ser Arg Gly Arg Thr Met Asn Ile
    170                 175                 180

aag tct gca aca tgg aag gta ttg cac tgc aca ggc cac att cac gta      628
Lys Ser Ala Thr Trp Lys Val Leu His Cys Thr Gly His Ile His Val
185                 190                 195                 200

tat gat acc aac agt aac caa cct cag tgt ggg tat aag aaa cca cct      676
Tyr Asp Thr Asn Ser Asn Gln Pro Gln Cys Gly Tyr Lys Lys Pro Pro
                205                 210                 215

atg acc tgc ttg gtg ctg att tgt gaa ccc att cct cac cca tca aat      724
Met Thr Cys Leu Val Leu Ile Cys Glu Pro Ile Pro His Pro Ser Asn
            220                 225                 230

att gaa att cct tta gat agc aag act ttc ctc agt cga cac agc ctg      772
Ile Glu Ile Pro Leu Asp Ser Lys Thr Phe Leu Ser Arg His Ser Leu
        235                 240                 245

gat atg aaa ttt tct tat tgt gat gaa aga att acc gaa ttg atg gga      820
Asp Met Lys Phe Ser Tyr Cys Asp Glu Arg Ile Thr Glu Leu Met Gly
    250                 255                 260

tat gag cca gaa gaa ctt tta ggc cgc tca att tat gaa tat tat cat      868
Tyr Glu Pro Glu Glu Leu Leu Gly Arg Ser Ile Tyr Glu Tyr Tyr His
265                 270                 275                 280

gct ttg gac tct gat cat ctg acc aaa act cat cat gat atg ttt act      916
Ala Leu Asp Ser Asp His Leu Thr Lys Thr His His Asp Met Phe Thr
                285                 290                 295

aaa gga caa gtc acc aca gga cag tac agg atg ctt gcc aaa aga ggt      964
Lys Gly Gln Val Thr Thr Gly Gln Tyr Arg Met Leu Ala Lys Arg Gly
            300                 305                 310

gga tat gtc tgg gtt gaa act caa gca act gtc ata tat aac acc aag     1012
Gly Tyr Val Trp Val Glu Thr Gln Ala Thr Val Ile Tyr Asn Thr Lys
        315                 320                 325
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aat tct caa cca cag tgc att gta tgt gtg aat tac gtt gtg agt ggt     1060
Asn Ser Gln Pro Gln Cys Ile Val Cys Val Asn Tyr Val Val Ser Gly
    330                 335                 340

att att cag cac gac ttg att ttc tcc ctt caa caa aca gaa tgt gtc     1108
Ile Ile Gln His Asp Leu Ile Phe Ser Leu Gln Gln Thr Glu Cys Val
345                 350                 355                 360

ctt aaa ccg gtt gaa tct tca gat atg aaa atg act cag cta ttc acc     1156
Leu Lys Pro Val Glu Ser Ser Asp Met Lys Met Thr Gln Leu Phe Thr
                365                 370                 375

aaa gtt gaa tca gaa gat aca agt agc ctc ttt gac aaa ctt aag aag     1204
Lys Val Glu Ser Glu Asp Thr Ser Ser Leu Phe Asp Lys Leu Lys Lys
            380                 385                 390

gaa cct gat gct tta act ttg ctg gcc cca gcc gct gga gac aca atc     1252
Glu Pro Asp Ala Leu Thr Leu Leu Ala Pro Ala Ala Gly Asp Thr Ile
        395                 400                 405

ata tct tta gat ttt ggc agc aac gac aca gaa act gat gac cag caa     1300
Ile Ser Leu Asp Phe Gly Ser Asn Asp Thr Glu Thr Asp Asp Gln Gln
    410                 415                 420

ctt gag gaa gta cca tta tat aat gat gta atg ctc ccc tca ccc aac     1348
Leu Glu Glu Val Pro Leu Tyr Asn Asp Val Met Leu Pro Ser Pro Asn
425                 430                 435                 440

gaa aaa tta cag aat ata aat ttg gca atg tct cca tta ccc acc gct     1396
Glu Lys Leu Gln Asn Ile Asn Leu Ala Met Ser Pro Leu Pro Thr Ala
                445                 450                 455

gaa acg cca aag cca ctt cga agt agt gct gac cct gca ctc aat caa     1444
Glu Thr Pro Lys Pro Leu Arg Ser Ser Ala Asp Pro Ala Leu Asn Gln
            460                 465                 470

gaa gtt gca tta aaa tta gaa cca aat cca gag tca ctg gaa ctt tct     1492
Glu Val Ala Leu Lys Leu Glu Pro Asn Pro Glu Ser Leu Glu Leu Ser
        475                 480                 485

ttt acc atg ccc cag att cag gat cag aca cct agt cct tcc gat gga     1540
Phe Thr Met Pro Gln Ile Gln Asp Gln Thr Pro Ser Pro Ser Asp Gly
    490                 495                 500

agc act aga caa agt tca cct gag cct aat agt ccc agt gaa tat tgt     1588
Ser Thr Arg Gln Ser Ser Pro Glu Pro Asn Ser Pro Ser Glu Tyr Cys
505                 510                 515                 520

ttt tat gtg gat agt gat atg gtc aat gaa ttc aag ttg gaa ttg gta     1636
Phe Tyr Val Asp Ser Asp Met Val Asn Glu Phe Lys Leu Glu Leu Val
                525                 530                 535

gaa aaa ctt ttt gct gaa gac aca gaa gca aag aac cca ttt tct act     1684
Glu Lys Leu Phe Ala Glu Asp Thr Glu Ala Lys Asn Pro Phe Ser Thr
            540                 545                 550

cag gac aca gat tta gac ttg gag atg tta gct ccc tat atc cca atg     1732
Gln Asp Thr Asp Leu Asp Leu Glu Met Leu Ala Pro Tyr Ile Pro Met
        555                 560                 565

gat gat gac ttc cag tta cgt tcc ttc gat cag ttg tca cca tta gaa     1780
Asp Asp Asp Phe Gln Leu Arg Ser Phe Asp Gln Leu Ser Pro Leu Glu
    570                 575                 580

agc agt tcc gca agc cct gaa agc gca agt cct caa agc aca gtt aca     1828
Ser Ser Ser Ala Ser Pro Glu Ser Ala Ser Pro Gln Ser Thr Val Thr
585                 590                 595                 600

gta ttc cag cag act caa ata caa gaa cct act gct aat gcc acc act     1876
Val Phe Gln Gln Thr Gln Ile Gln Glu Pro Thr Ala Asn Ala Thr Thr
                605                 610                 615

acc act gcc acc act gat gaa tta aaa aca gtg aca aaa gac cgt atg     1924
Thr Thr Ala Thr Thr Asp Glu Leu Lys Thr Val Thr Lys Asp Arg Met
            620                 625                 630

gaa gac att aaa ata ttg att gca tct cca tct cct acc cac ata cat     1972
Glu Asp Ile Lys Ile Leu Ile Ala Ser Pro Ser Pro Thr His Ile His
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        635                 640                 645

aaa gaa act act agt gcc aca tca tca cca tat aga gat act caa agt     2020
Lys Glu Thr Thr Ser Ala Thr Ser Ser Pro Tyr Arg Asp Thr Gln Ser
    650                 655                 660

cgg aca gcc tca cca aac aga gca gga aaa gga gtc ata gaa cag aca     2068
Arg Thr Ala Ser Pro Asn Arg Ala Gly Lys Gly Val Ile Glu Gln Thr
665                 670                 675                 680

gaa aaa tct cat cca aga agc cct aac gtg tta tct gtc gct ttg agt     2116
Glu Lys Ser His Pro Arg Ser Pro Asn Val Leu Ser Val Ala Leu Ser
                685                 690                 695

caa aga act aca gtt cct gag gaa gaa cta aat cca aag ata cta gct     2164
Gln Arg Thr Thr Val Pro Glu Glu Glu Leu Asn Pro Lys Ile Leu Ala
            700                 705                 710

ttg cag aat gct cag aga aag cga aaa atg gaa cat gat ggt tca ctt     2212
Leu Gln Asn Ala Gln Arg Lys Arg Lys Met Glu His Asp Gly Ser Leu
        715                 720                 725

ttt caa gca gta gga att gga aca tta tta cag cag cca gac gat cat     2260
Phe Gln Ala Val Gly Ile Gly Thr Leu Leu Gln Gln Pro Asp Asp His
    730                 735                 740

gca gct act aca tca ctt tct tgg aaa cgt gta aaa gga tgc aaa tct     2308
Ala Ala Thr Thr Ser Leu Ser Trp Lys Arg Val Lys Gly Cys Lys Ser
745                 750                 755                 760

agt gaa cag aat gga atg gag caa aag aca att att tta ata ccc tct     2356
Ser Glu Gln Asn Gly Met Glu Gln Lys Thr Ile Ile Leu Ile Pro Ser
                765                 770                 775

gat tta gca tgt aga ctg ctg ggg caa tca atg gat gaa agt gga tta     2404
Asp Leu Ala Cys Arg Leu Leu Gly Gln Ser Met Asp Glu Ser Gly Leu
            780                 785                 790

cca cag ctg acc agt tat gat tgt gaa gtt aat gct cct ata caa ggc     2452
Pro Gln Leu Thr Ser Tyr Asp Cys Glu Val Asn Ala Pro Ile Gln Gly
        795                 800                 805

agc aga aac cta ctg cag ggt gaa gaa tta ctc aga gct ttg gat caa     2500
Ser Arg Asn Leu Leu Gln Gly Glu Glu Leu Leu Arg Ala Leu Asp Gln
    810                 815                 820

gtt aac tga gctttttctt aatttcattc ctttttttgg acactggtgg             2549
Val Asn
825

ctcactacct aaagcagtct atttatattt tctacatcta attttagaag cctggctaca   2609

atactgcaca aacttggtta gttcaatttt tgatcccctt tctacttaat ttacattaat   2669

gctctttttt agtatgttct ttaatgctgg atcacagaca gctcattttc tcagtttttt   2729

ggtatttaaa ccattgcatt gcagtagcat cattaattaa aaaatgcacc tttttattta   2789

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aatttttgta agaaggcagt aacctttcat catgatcata ggcagttgaa aaatttttac   2909

acctttttttt tcacaaattt tacataaata ataatgcttt gccagcagta cgtggtagcc   2969

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ggaacatgac attgttaatc atataataat gattcttaaa tgctgtatgg tttattattt   3149

aaatgggtaa agccatttac ataatataga aagatatgca tatatctaga aggtatgtgg   3209

catttatttg gataaaattc tcaattcaga gaaatcaaat ctgatgtttc tatagtcact   3269

ttgccagctc aaaagaaaac aataccctat gtagttgtgg aagtttatgc taatattgtg   3329

taactgatat taaacctaaa tgttctgcct accctgttgg tataaagata ttttgagcag   3389

actgtaaaca agaaaaaaaa aaaatcatgc attcttagca aaattgccta gtatgttaat   3449
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ttgctcaaaa tacaatgttt gattttatgc actttgtcgc tattaacatc ctttttttca 3509

tgtagatttc aataattgag taattttaga agcattattt taggaatata tagttgtcaa 3569

aaacagtaaa tatcttgttt tttctatgta cattgtacaa atttttcatt ccttttgctc 3629

tttgtggttg gatctaacac taactgtatt gttttgttac atcaaataaa catcttctgt 3689

ggaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaa 3736

<210> SEQ ID NO 2
<211> LENGTH: 826
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 2

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Arg Arg Lys Glu Lys Ser Arg Asp Ala Ala Arg Ser Arg Arg Ser Lys
20 25 30

Glu Ser Glu Val Phe Tyr Glu Leu Ala His Gln Leu Pro Leu Pro His
35 40 45

Asn Val Ser Ser His Leu Asp Lys Ala Ser Val Met Arg Leu Thr Ile
50 55 60

Ser Tyr Leu Arg Val Arg Lys Leu Leu Asp Ala Gly Asp Leu Asp Ile
65 70 75 80

Glu Asp Asp Met Lys Ala Gln Met Asn Cys Phe Tyr Leu Lys Ala Leu
85 90 95

Asp Gly Phe Val Met Val Leu Thr Asp Asp Gly Asp Met Ile Tyr Ile
100 105 110

Ser Asp Asn Val Asn Lys Tyr Met Gly Leu Thr Gln Phe Glu Leu Thr
115 120 125

Gly His Ser Val Phe Asp Phe Thr His Pro Cys Asp His Glu Glu Met
130 135 140

Arg Glu Met Leu Thr His Arg Asn Gly Leu Val Lys Lys Gly Lys Glu
145 150 155 160

Gln Asn Thr Gln Arg Ser Phe Phe Leu Arg Met Lys Cys Thr Leu Thr
165 170 175

Ser Arg Gly Arg Thr Met Asn Ile Lys Ser Ala Thr Trp Lys Val Leu
180 185 190

His Cys Thr Gly His Ile His Val Tyr Asp Thr Asn Ser Asn Gln Pro
195 200 205

Gln Cys Gly Tyr Lys Lys Pro Pro Met Thr Cys Leu Val Leu Ile Cys
210 215 220

Glu Pro Ile Pro His Pro Ser Asn Ile Glu Ile Pro Leu Asp Ser Lys
225 230 235 240

Thr Phe Leu Ser Arg His Ser Leu Asp Met Lys Phe Ser Tyr Cys Asp
245 250 255

Glu Arg Ile Thr Glu Leu Met Gly Tyr Glu Pro Glu Glu Leu Leu Gly
260 265 270

Arg Ser Ile Tyr Glu Tyr Tyr His Ala Leu Asp Ser Asp His Leu Thr
275 280 285

Lys Thr His His Asp Met Phe Thr Lys Gly Gln Val Thr Thr Gly Gln
290 295 300

Tyr Arg Met Leu Ala Lys Arg Gly Gly Tyr Val Trp Val Glu Thr Gln
305 310 315 320

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Ala Thr Val Ile Tyr Asn Thr Lys Asn Ser Gln Pro Gln Cys Ile Val
                325                 330                 335

Cys Val Asn Tyr Val Val Ser Gly Ile Ile Gln His Asp Leu Ile Phe
            340                 345                 350

Ser Leu Gln Gln Thr Glu Cys Val Leu Lys Pro Val Glu Ser Ser Asp
        355                 360                 365

Met Lys Met Thr Gln Leu Phe Thr Lys Val Glu Ser Glu Asp Thr Ser
    370                 375                 380

Ser Leu Phe Asp Lys Leu Lys Lys Glu Pro Asp Ala Leu Thr Leu Leu
385                 390                 395                 400

Ala Pro Ala Ala Gly Asp Thr Ile Ile Ser Leu Asp Phe Gly Ser Asn
                405                 410                 415

Asp Thr Glu Thr Asp Asp Gln Gln Leu Glu Glu Val Pro Leu Tyr Asn
            420                 425                 430

Asp Val Met Leu Pro Ser Pro Asn Glu Lys Leu Gln Asn Ile Asn Leu
        435                 440                 445

Ala Met Ser Pro Leu Pro Thr Ala Glu Thr Pro Lys Pro Leu Arg Ser
    450                 455                 460

Ser Ala Asp Pro Ala Leu Asn Gln Glu Val Ala Leu Lys Leu Glu Pro
465                 470                 475                 480

Asn Pro Glu Ser Leu Glu Leu Ser Phe Thr Met Pro Gln Ile Gln Asp
                485                 490                 495

Gln Thr Pro Ser Pro Ser Asp Gly Ser Thr Arg Gln Ser Ser Pro Glu
            500                 505                 510

Pro Asn Ser Pro Ser Glu Tyr Cys Phe Tyr Val Asp Ser Asp Met Val
        515                 520                 525

Asn Glu Phe Lys Leu Glu Leu Val Glu Lys Leu Phe Ala Glu Asp Thr
    530                 535                 540

Glu Ala Lys Asn Pro Phe Ser Thr Gln Asp Thr Asp Leu Asp Leu Glu
545                 550                 555                 560

Met Leu Ala Pro Tyr Ile Pro Met Asp Asp Asp Phe Gln Leu Arg Ser
                565                 570                 575

Phe Asp Gln Leu Ser Pro Leu Glu Ser Ser Ser Ala Ser Pro Glu Ser
            580                 585                 590

Ala Ser Pro Gln Ser Thr Val Thr Val Phe Gln Gln Thr Gln Ile Gln
        595                 600                 605

Glu Pro Thr Ala Asn Ala Thr Thr Thr Thr Ala Thr Thr Asp Glu Leu
    610                 615                 620

Lys Thr Val Thr Lys Asp Arg Met Glu Asp Ile Lys Ile Leu Ile Ala
625                 630                 635                 640

Ser Pro Ser Pro Thr His Ile His Lys Glu Thr Thr Ser Ala Thr Ser
                645                 650                 655

Ser Pro Tyr Arg Asp Thr Gln Ser Arg Thr Ala Ser Pro Asn Arg Ala
            660                 665                 670

Gly Lys Gly Val Ile Glu Gln Thr Glu Lys Ser His Pro Arg Ser Pro
        675                 680                 685

Asn Val Leu Ser Val Ala Leu Ser Gln Arg Thr Thr Val Pro Glu Glu
    690                 695                 700

Glu Leu Asn Pro Lys Ile Leu Ala Leu Gln Asn Ala Gln Arg Lys Arg
705                 710                 715                 720

Lys Met Glu His Asp Gly Ser Leu Phe Gln Ala Val Gly Ile Gly Thr
                725                 730                 735

Leu Leu Gln Gln Pro Asp Asp His Ala Ala Thr Thr Ser Leu Ser Trp
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            740                 745                 750

Lys Arg Val Lys Gly Cys Lys Ser Ser Glu Gln Asn Gly Met Glu Gln
        755                 760                 765

Lys Thr Ile Ile Leu Ile Pro Ser Asp Leu Ala Cys Arg Leu Leu Gly
    770                 775                 780

Gln Ser Met Asp Glu Ser Gly Leu Pro Gln Leu Thr Ser Tyr Asp Cys
785                 790                 795                 800

Glu Val Asn Ala Pro Ile Gln Gly Ser Arg Asn Leu Leu Gln Gly Glu
                805                 810                 815

Glu Leu Leu Arg Ala Leu Asp Gln Val Asn
            820                 825
```

What is claimed is:

**1**. A substantially purified hypoxia inducible factor-1 (HIF-1), having a sequence comprising amino acid residues 1–391 and 429–826 of SEQ ID NO: 2; amino acid residues 1–391 and 469–826 of SEQ ID NO:2; amino acid residues of 1–391 and 494–826 of SEQ ID NO: 2; amino acid residues 1–391 and 508–826 of SEQ ID NO: 2; amino acid residues 1–391 and 512–826 of SEQ ID NO:2; or amino acid residues 1–391 and 517–826 of SEQ ID NO:2, wherein amino acid 551 is changed from a serine to any other amino acid, and amino acid 552 is changed from a threonine to any other amino acid.

**2**. The hypoxia inducible factor-1 of claim **1**, wherein amino acid 551 is a glycine.

**3**. The hypoxia inducible factor-1 of claim **1**, wherein amino acid 552 is an alanine.

**4**. The hypoxia inducible factor-1 of claim **1**, further comprising a deletion of amino acids 576–785.

**5**. An isolated nucleic acid sequence encoding a human hypoxia inducible factor-1 (HIF-1) of claim **1**.

**6**. The nucleic acid of claim **5**, further comprising an expression control sequence operatively linked to a nucleic acid encoding a hypoxia inducible factor-1 (HIF-1).

**7**. The nucleic acid sequence of claim **6**, wherein the expression control sequence is a promoter.

**8**. The nucleic acid sequence of claim **7**, wherein the promoter is tissue specific.

**9**. An expression vector containing the polynucleotide of claim **5**.

**10**. The vector of claim **9**, wherein the vector is a plasmid.

**11**. The vector of claim **9**, wherein the vector is a viral vector.

**12**. The vector of claim **11**, wherein the vector is a retroviral vector.

**13**. An isolated host cell containing the vector of claim **9**.

**14**. An isolated host cell of claim **13**, wherein the cell is a eukaryotic cell.

**15**. An isolated host cell of claim **13**, wherein the cell is a prokaryotic cell.

**16**. An antibody which selectively binds to the polypeptide of claim **1**.

**17**. The antibody of claim **16**, wherein the antibody is monoclonal.

**18**. The antibody of claim **16**, wherein the antibody is polyclonal.

* * * * *